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EDITORIAL

Immune response to *H pylori*

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Abstract

The gastric mucosa separates the underlying tissue from the vast array of antigens that traffic through the stomach lumen. While the extreme pH of this environment is essential in aiding the activation of enzymes and food digestion, it also renders the gastric epithelium free from bacterial colonization, with the exception of one important human pathogen, *H pylori*. This bacterium has developed mechanisms to survive the harsh environment of the stomach, actively move through the mucosal layer, attach to the epithelium, evade immune responses, and achieve persistent colonization. While a hallmark of this infection is a marked inflammatory response with the infiltration of various immune cells into the infected gastric mucosa, the host immune response is unable to clear the infection and may actually contribute to the associated pathogenesis. Here, we review the host responses involved during infection with *H pylori* and how they are influenced by this bacterium.

Key words: *H pylori*; Immune response; T cell; Dendritic cells

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INTRODUCTION

H pylori is one of the most common human pathogens, since it infects the gastric mucosa of about 50% of the world's population. The majority of infections are asymptomatic, making the infection lifelong without effective bacterial eradication. The clinical magnitude of this bacterium has become accepted rather recently. *H pylori* has been recognized as the causal agent for chronic gastritis and gastric and duodenal ulcers. Additionally, epidemiological and statistical studies associated the infection with a higher risk of gastric malignancy leading the World Health Organization International Agency for Research in Cancer to categorize *H pylori* as a class I carcinogen.

The bacteria induce a host immune response, but the persistence of the infection suggests that the response is not effective in eliminating the infection. Furthermore, multiple lines of evidence suggest that the immune response contributes to the pathogenesis associated with the infection. As a result, the immune response induced by *H pylori* is a subject of continuous study that has encouraged numerous questions. The inability of the host response to clear infections with *H pylori* could reflect down-regulatory mechanisms that limit the resulting immune responses to prevent harmful inflammation as a means to protect the host. Consequently, the chronic immune response induced may be inadequate or misdirected, and could thus afford a colonization advantage for the bacteria by providing improved availability of adhesion places. An example of this is the resulting increase in class II major histocompatibility complex (MHC) and CD74, induced by IFN-γ and IL-8, that are used as receptors by *H pylori*[1-3].

H pylori has been shown to employ multiple mechanisms to antagonize, impair, or subvert host responses[4]. For instance, *H pylori* has been noted to inhibit macrophage nitric oxide production and phagocytosis[5]. Another mechanism where *H pylori* can down-regulate the immune response is through its VacA virulence factor. This cytotoxin can interfere with the processing and presentation of antigens by antigen-presenting cells (APCs)[6], and can also inhibit T cell activation through interference of the calcineurin-associated IL-2 signaling pathway[7]. These and multiple other observations on the nature of the immune response during *H pylori* infection have led to models that help explain how the bacteria could persist in the gastric environment by generating a non-effective immune response. The ineffective response, together with the host factors, determines the severity of the disease.

HUMORAL RESPONSE

Nearly everyone infected with *H pylori* develops specific antibodies, which are found in serum and in gastric

aspirates or extracts of stomach. Accordingly, elevated titers of IgG and IgA antibodies directed at membrane proteins (MP), flagelin, urease, lipopolysaccharide (LPS) and *H pylori* adhesin A (HpaA) have been reported in patients infected with *H pylori*[8,9]. Yet, those titers do not differ between asymptomatic patients and patients with duodenal ulcers. IgM- and IgA-producing cells in biopsies from the antral region of the *H pylori*-infected patients' stomaches were 40- to 50-fold higher in frequency than in non-infected subjects. However, IgG-producing cell numbers are the same for non-infected and infected *H pylori* subjects. Those results suggest that the infection induces a large recruitment of immune cells into the gastric mucosa, particularly IgA-producing cells. A recent immunoproteome analysis compared individual sera from *H pylori*-positive patients suffering from gastric adenocarcinoma or duodenal ulcer with a pool of five sera from *H pylori*-negative patients to detect antigenic proteins from three separate *H pylori* strains[10]. That study recognized 30 antigens detected by *H pylori* positive sera, nine of these were newly identified and 21 established previously. The study established the presence of antigens related to specific disease. Interestingly, cancer sera provided stronger immunoreactivity while a similar study suggested that sera from ulcer patients have more anti-*H pylori* antibodies than sera from gastritis patients[11].

Due to the plasticity of the *H pylori* genome as well as the phase variation that the bacteria present in its LPS, specifically mimic Lewis antigens, 20% to 30% of the people infected with *H pylori* develop autoantibodies, with most of them specific to the gastric proton pump located in the parietal cells. These antibodies may block pump function, leading to achlorhydria associated with the infection, which contributes to the gastric damage seen during infection.

T-CELL RESPONSE

H pylori induce the recruitment of $CD4^+$ and $CD8^+$ T-cells into the gastric mucosa, but there appears to be preferential activation of $CD4^+$ cells rather than $CD8^+$ cells[12]. Several studies have noted that the T helper cell response to *H pylori* is polarized, since $CD4^+$ T cells in the gastric mucosa of infected individuals produce the Th1 cytokines, interleukin (IL)-12 and interferon (IFN)-γ, whereas IL-4, a Th2 cytokine, production by these T cells is absent[13,14]. A recent study by Amedei *et al*[15] suggested that *H pylori* neutrophil-activating protein (HP-NAP) contributes to this Th1-polarized T cell response in the gastric mucosa of *H pylori*-infected patients. In that study, addition of HP-NAP to antigen-induced T cell lines in culture resulted in a shift from a predominant Th2 to a Th1 phenotype of specific T cells.

Another subset of $CD4^+$ T cells are T regulatory cells, which produce IL-10 and transforming growth factor (TGF)-β[16]. While there is a demonstrated infiltration of T cells in the gastric mucosa and most of those are $CD4^+$ T cells with markers of activation, various studies have tried to address the inefficiency of the host response in clearing the infection. Different studies have demonstrated that *H pylori* infection can decrease T cell responses as well as induce T cell anergy[12]. $CD45RO^+$ memory T cells as well as activated $CD69^+$ and $CD25^+$ T cells are increased in the antral lamina propria of infected subjects[17]. Memory T cells isolated from peripheral blood from infected people responded less to stimulation with *H pylori* antigens than cells isolated from non-infected subjects[12,18,19]. These results suggested the presence of regulatory T (T reg) cells, $CD4^+$ and $CD25^+$ in the peripheral blood of *H pylori* infected individuals, which could inhibit the response of $CD4^+$ T cells to *H pylori*. This notion was supported by observations that a higher responsiveness was obtained after depletion of *H pylori*-specific T reg cells[20,12]. Hence, these observations may help explain the inability of the host response to eliminate the infection due to the activation of T reg cells, which were recently reported to be increased in the gastric mucosa of *H pylori*-infected individuals and were described as $CD4^+$, $CD25^{high}$ and $FOXP3^+$. Such cells may simultaneously reduce mucosal damage mediated by T cells as well as reduce specific T cell responses, possibly by reducing activation of IFN-γ-producing $CD4^+$ T cells that can be effective in protection against the infection with these bacteria. Figure 1 illustrates the presence of the various T cell populations in the infected gastric mucosa and how they may interact with one another and with other resident cells.

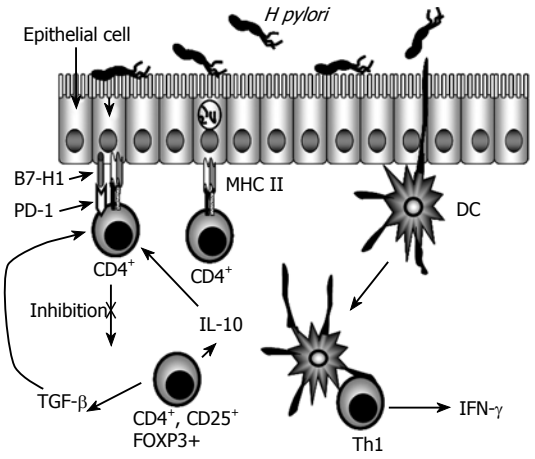

Figure 1 Regulation of $CD4^+$ T Cells During *H pylori* Infection. $CD4^+$ T cell numbers increase in the gastric lamina propria of individuals infected with *H pylori*. These cells are predominantly Th1 cells characterized by their production of IFN-γ. Because the epithelium separates *H pylori* from $CD4^+$ T cells, and also expresses key proteins associated with antigen presenting cells, the gastric epithelium, in addition to dendritic cells, could be involved in the presentation of antigens to these $CD4^+$ T cells. The expression of inhibitory B7 related molecules along with $CD4^+$ T cells with a regulatory T cell phenotype could be playing a role in limiting the function of effector $CD4^+$ T cells.

The role of $CD8^+$ T cells in the gastric mucosa of *H pylori*-infected individuals is less clear than that of the $CD4^+$ T cells. Although their numbers are also increased, the $CD8^+$ T cells that are found in the infected tissue are thought to be intraepithelial lymphocytes. Their contribution to the local response is in the form of IFN-γ production, which in turn helps increase class II MHC molecule expression on adjacent cells. A recent report by Azem and colleagues showed that *H pylori*-reactive $CD8^+$ T cells can be efficiently stimulated by *H pylori* antigen-pulsed B cells and DCs, and that most of the $CD8^+$ T cells in the infected gastric mucosa are memory T cells[21].

ANTIGEN PRESENTING CELLS IN THE GASTRIC MUCOSA

The activation of $CD4^+$ T cells requires their effective cross talk with cells that express class II MHC molecules, which are classically referred to as antigen presenting cells (APC). Conventional APC include macrophages, dendritic cells and B cells. The role of these cells in the adaptive response is to internalize foreign antigens and present them in the form of peptides bound to class II MHC molecules to the T cells. The infected gastric mucosa contains a significant macrophage population that produces nitric oxide, IL-6, IL-1β, TNF-α, and IL-12 that help drive a T helper 1 response responsible for the production of IFN-γ, and little or no IL-4 and IL-5[22,23]. Although in smaller numbers, dendritic cells are also present and respond to *H pylori* with the production of IL-6, IL-8, IL-10 and IL-12, and have increased expression of CD80, CD83, CD86, and HLA-DR as a result of their stimulation with *H pylori*[24].

For efficient T cell activation, T cells require not only the T cell antigen receptor (TCR)-mediated signaling, but also costimulatory signals provided by APC[25]. The B7 family of molecules provides signals that are critical for both stimulating and inhibiting T cell activation. Engagement of CD28 by CD80 (B7-1) and CD86 (B7-2) stimulates and sustains T cell responses, whereas engagement of CTLA-4 by the same ligand inhibits T cell responses[26]. Recently, several new members of the B7 family have been identified. B7-H2 (homologue 2 also known as GL50, B7h, B7RP-1 and LICOS) has been identified as a ligand for the CD28 family member ICOS (inducible T-cell co-stimulator). Two additional B7 family members, Programmed Death-Ligand 1, PD-L1 (B7-H1) and PD-L2 (B7-DC) bind to the receptor Programmed Death-1 (PD-1) and their interaction down regulates T cell activation[27]. PD-1 is a type I transmembrane receptor expressed on activated T and B cells. Like CTLA-4, PD-1 contains an immunoreceptor tyrosine based inhibitory (ITIM) motif in its cytoplasmic region and acts as a negative regulator of lymphocyte function via multiple mechanisms, including cell-cycle inhibition and apoptosis. The literature suggests that there is another unidentified receptor for B7-H1 and B7-DC whose function has yet to be determined. Two other receptors of the B7 family are B7-H3 and B7-H4 (also known as B7S1 and B7x); however, their receptors and functions are still unclear[28-30]. In an attempt to examine whether changes in the expression of these novel B7 family members could contribute to the hyporesponsiveness of T cells in the infected gastric mucosa, we examined by real-time PCR the expression of the message for these molecules in gastric biopsies and observed the expression of B7-H1, B7-DC, and B7-H3. Since, as discussed below, the epithelium is exposed to both *H pylori* and T cells in the lamina propria, we examined the epithelium for these molecules and detected their expression by PCR and by Western blot analysis. B7-H1 was the most prominent coinhibitory molecule of the B7 family whose expression was induced following *H pylori* infection. More interesting, epithelial cells in gastric biopsies infected with *H pylori* showed higher B7-H1 expression compared with uninfected samples[31]. Gastric epithelial cells were found to constitutively express B7-H1, and the level of expression increased significantly during infection. T cells cocultured with gastric epithelial cells exposed to *H pylori* had a lower proliferation index, IL-2 secretion, and CD69 expression in response to activation via CD3. However, blockage of B7-H1 with specific anti-B7-H1 antibodies restored the responses to levels close to those of T cells not cocultured with gastric epithelial cells. This may represent a novel mechanism of immune avoidance used by *H pylori*, which involves the induction of coinhibitory molecule expression on gastric epithelial cells by the bacterium.

Recent elegant studies by Anderson and colleagues showed the importance of CTLA-4 in establishing T cell anergy during *H pylori* infection in a murine model. In this model of *H pylori* infection, the mice that received anti-CTLA-4 Fabs responded to an *H pylori* challenge with much greater inflammation and drastically decreased bacterial numbers. Their results suggested that CTLA-4 engagement may represent yet another mechanism of inactivation of *H pylori*-specific T cells during *H pylori* infection, which could in turn contribute to the chronicity of this infection[32].

While direct interaction between APC and T cells represent the traditional mechanism leading to T cell activation, another mechanism that is under active investigation involves exosomes secreted by APC. Exosomes are small membrane vesicles derived from late endosomes, which are released into the extracellular membrane and interact with membranes of other cells at a relative distance. Exosomes secreted by APC carry class I and II MHC molecules, costimulatory molecules, and adhesins. Thus, they have immunomodulatory capacity, such as in the activation of naïve T cells[33]. They have been shown to stimulate T cells *in vitro* and to induce anti-tumor responses *in vivo*[34,35]. While they are not yet characterized in the context of the T cell response to *H pylori*, their contribution in modulating the local response has to be considered.

Human dendritic cells have been shown to produce IL-8, IL-10, and IL-12 in response to *H pylori* as well as to purified *H pylori* antigens[36,37]. Thus, *H pylori* can bind to the dendritic cell receptor DC-specific ICAM-3-grabbing nonintegrin (SIGN) through the blood group Lewis X antigen present in its LPS[38]. This interaction can alter the T helper balance and favor pathogen persistence. Also, in monocytes, urease and HSP60 have been shown to be potent activators of proinflammatory cytokines via NF-κB activation[39,40].

THE GASTRIC EPITHELIUM AS AN ACTIVE PLAYER IN THE MUCOSAL RESPONSE

In terms of providing protection, the gastric epithelium has typically been regarded as a physical barrier; however, multiple studies have provided evidence to suggest that the gastric epithelium plays a key role in the inflammatory and immune responses induced by *H pylori*. The epithelium is the only cell phenotype in the gastric mucosa that is in direct contact with the pathogen. This feature places the

epithelium in a strategic situation to interact with *H pylori* and with the immune elements in the lamina propria. There is strong evidence to suggest that the gastric epithelium is an active player in the response while performing functions associated with antigen presenting cells[41,42]. In addition, it is well documented that the epithelium has the ability to produce cytokines that trigger the recruitment of inflammatory cells into the gastric lamina propria[1]. The production of IL-8 in response to *H pylori* infection is one of the first epithelial responses. This chemokine recruits immunological components into the gastric mucosa from the periphery, particularly polymorphonuclear cells, which contribute to epithelial damage[43]. Macrophages also contribute to epithelial damage by producing nitric oxide in response to *H pylori* urease leading to the induction of additional inflammatory mediators[44]. However, the bacteria produce an arginase encoded by the gene *rocF* that competes with the NOS for L-arginine and converts this to urea and L-ornithine rather than NO[45].

One of the major mechanisms of IL-8 induction by epithelial cells is through the injection of CagA into gastric epithelial cells by a type Ⅳ secretion system[46]. This system releases CagA into the epithelial cells cytosol inducing cell proliferation and IL-8 production[47]. Our group has recently described the interaction of *H pylori* with CD74 on gastric epithelial cells (GEC) leading to the production of IL-8, *via* NF-κB activation[1]. Interestingly, IL-8 induced by *H pylori*, in addition to its effect in the recruitment of inflammatory cells, also acts in an autocrine manner and induces further expression of CD74[2]. This, in turn, suggests that *H pylori* has the ability to induce the increased expression of receptors on the host epithelium to enhance colonization and the stimulation of proinflammatory responses. As part of the inflammatory response, we noted that the *H pylori*-infected gastric epithelial cells produce macrophage migration inhibitory factor (MIF), which is an important cytokine that bridges the innate and adaptive immune responses[48]. The production of MIF was found to be dependent on CagA, since CagA-deficient mutant *H pylori* strains had a significantly reduced ability to stimulate MIF production.

Some of the interactions of the epithelium with *H pylori* can be detrimental to the integrity of the epithelium. For instance, we have shown that *H pylori* use class II MHC as receptors on GECs, and this interaction leads to apoptosis[3]. This interaction is mediated *via H pylori* urease. It has also been reported that cag genes may up-regulate Fas ligand (FasL) expression leading T cells to undergo apoptosis[49]. Thus, the contribution of the gastric epithelium in influencing the adaptive response by expressing molecules that either directly or indirectly limit T cell activity has to be considered in our ongoing efforts to understand the host response to *H pylori*.

THE INNATE RESPONSE TO *H PYLORI*

Other potential interactions that lead to production of pro-inflammatory cytokines include that of *H pylori* with toll-like receptors (TLR) expressed by epithelial cells. It has been reported that gastric epithelial cells express TLR2, TLR4, TLR5, and TLR9[50-53] that interact respectively with lipoproteins, LPS, flagellin, and CpG motifs. The expression of those receptors by epithelial cells is of importance in innate immunity against *H pylori*. Since these innate receptors may elicit cytokine secretion when they bind their ligands, they may have an indirect effect in the subsequent adaptive response through the enhancement of processing and presentation of antigen by host cells. However, it has been demonstrated that *H pylori* LPS has a 500-1000 fold lower endotoxic activity than LPS from *S. typhimurium* and *E. coli*[54,55]. This low stimulatory potential can be attributed to the phosphorylation pattern and the LPS' lipid A acylation[55]. In addition, *H pylori* LPS has low binding affinity to LPS-binding protein (LBP) and in consequence, has a lower transfer rate to CD14 present in macrophages and monocytes[56].

Another ligand for TLR receptors on the epithelium is *H pylori* flagellin. This flagellin contains different amino acids than that of other bacteria in the TLR5 recognition site, as well as having a compensatory mutation that preserves bacterial motility. Those differences avoid the recognition of flagellin by TLR5[57]. *H pylori* also avoids recognition by TLR9, which is the receptor for unmethylated CpG motif present in bacteria and viruses. Since *H pylori* DNA shows a high rate of methylation, it can evade the recognition of its DNA by TLR9.

Mast cells represent another innate cell phenotype that is found within the *H pylori*-infected gastric mucosa of humans and mice[58]. These cells represent an innate defense component that may kill bacteria through the release of proteases and other mediators. Additionally, an interesting observation was made in a recent study that showed that these cells can mediate bacterial clearance in vaccinated mice, and were suggested to do so *via* a cross talk with $CD4^+$ T cells[59].

In parallel with CagA, peptidoglycan (PGN) is also translocated into the epithelial cells by the *cag pathogenicity island* (PAI)-encoded type Ⅳ secretion system. Cag-PAI positive bacteria can induce the production of IL-8 *via* NF-κB in a manner that is CagA-independent by signaling through Nod1. Thus, *H pylori* PGN can interact with Nod1 and induce the activation of NF-κB[60].

CONCLUDING REMARKS

Infection with *H pylori* results in robust innate and acquired immune responses by the host, where the gastric epithelium represents a central player. Interaction of *H pylori* with the host epithelium results in the release of an array of chemokines and cytokines. Some of these factors are stimulated via the engagement of toll-like receptors or cell surface receptors, such as CD74. Also, injection of CagA via the bacterial type Ⅳ secretion system leads to NF-κB activation and the ensuing release of cytokines. The infected gastric mucosa is infiltrated by neutrophils and mononuclear cells as well as components of the acquired response, such as lymphocytes. A specific humoral response is also triggered during infection, as well as a T cell response that is skewed toward a Th1 cell response. In spite of these immune mechanisms, *H pylori* is not cleared because the bacteria seem to be equipped

with an array of mechanisms that allows them to evade or downregulate the host responses. Understanding these multiple mechanisms is a required step toward the development of any immune intervention strategies to protect from initial infection and to eliminate infections that are already established.

REFERENCES

1 **Beswick EJ**, Bland DA, Suarez G, Barrera CA, Fan X, Reyes VE. Helicobacter pylori binds to CD74 on gastric epithelial cells and stimulates interleukin-8 production. *Infect Immun* 2005; **73**: 2736-2743

2 **Beswick EJ**, Das S, Pinchuk IV, Adegboyega P, Suarez G, Yamaoka Y, Reyes VE. Helicobacter pylori-induced IL-8 production by gastric epithelial cells up-regulates CD74 expression. *J Immunol* 2005; **175**: 171-176

3 **Fan X**, Crowe SE, Behar S, Gunasena H, Ye G, Haeberle H, Van Houten N, Gourley WK, Ernst PB, Reyes VE. The effect of class II major histocompatibility complex expression on adherence of Helicobacter pylori and induction of apoptosis in gastric epithelial cells: a mechanism for T helper cell type 1-mediated damage. *J Exp Med* 1998; **187**: 1659-1669

4 **Ernst PB**, Peura DA, Crowe SE. The translation of Helicobacter pylori basic research to patient care. *Gastroenterology* 2006; **130**: 188-206; quiz 212-213

5 **Gobert AP**, Cheng Y, Wang JY, Boucher JL, Iyer RK, Cederbaum SD, Casero RA Jr, Newton JC, Wilson KT. Helicobacter pylori induces macrophage apoptosis by activation of arginase II. *J Immunol* 2002; **168**: 4692-4700

6 **Molinari M**, Salio M, Galli C, Norais N, Rappuoli R, Lanzavecchia A, Montecucco C. Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA. *J Exp Med* 1998; **187**: 135-140

7 **Gebert B**, Fischer W, Weiss E, Hoffmann R, Haas R. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 2003; **301**: 1099-1102

8 **Mattsson A**, Tinnert A, Hamlet A, Lönroth H, Bölin I, Svennerholm AM. Specific antibodies in sera and gastric aspirates of symptomatic and asymptomatic Helicobacter pylori-infected subjects. *Clin Diagn Lab Immunol* 1998; **5**: 288-293

9 **Mattsson A**, Quiding-Järbrink M, Lönroth H, Hamlet A, Ahlstedt I, Svennerholm A. Antibody-secreting cells in the stomachs of symptomatic and asymptomatic Helicobacter pylori-infected subjects. *Infect Immun* 1998; **66**: 2705-2712

10 **Mini R**, Bernardini G, Salzano AM, Renzone G, Scaloni A, Figura N, Santucci A. Comparative proteomics and immunoproteomics of Helicobacter pylori related to different gastric pathologies. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006; **833**: 63-79

11 **Haas G**, Karaali G, Ebermayer K, Metzger WG, Lamer S, Zimny-Arndt U, Diescher S, Goebel UB, Vogt K, Roznowski AB, Wiedenmann BJ, Meyer TF, Aebischer T, Jungblut PR. Immunoproteomics of Helicobacter pylori infection and relation to gastric disease. *Proteomics* 2002; **2**: 313-324

12 **Lundgren A**, Suri-Payer E, Enarsson K, Svennerholm AM, Lundin BS. Helicobacter pylori-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to H pylori in infected individuals. *Infect Immun* 2003; **71**: 1755-1762

13 **Bamford KB**, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK, Brooks EG, Graham DY, Reyes VE, Ernst PB. Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. *Gastroenterology* 1998; **114**: 482-492

14 **Haeberle HA**, Kubin M, Bamford KB, Garofalo R, Graham DY, El-Zaatari F, Karttunen R, Crowe SE, Reyes VE, Ernst PB. Differential stimulation of interleukin-12 (IL-12) and IL-10 by live and killed Helicobacter pylori in vitro and association of IL-12 production with gamma interferon-producing T cells in the human gastric mucosa. *Infect Immun* 1997; **65**: 4229-4235

15 **Amedei A**, Cappon A, Codolo G, Cabrelle A, Polenghi A, Benagiano M, Tasca E, Azzurri A, D'Elios MM, Del Prete G, de Bernard M. The neutrophil-activating protein of Helicobacter pylori promotes Th1 immune responses. *J Clin Invest* 2006; **116**: 1092-1101

16 **O'Garra A**, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004; **10**: 801-805

17 **Strömberg E**, Lundgren A, Edebo A, Lundin S, Svennerholm AM, Lindholm C. Increased frequency of activated T-cells in the Helicobacter pylori-infected antrum and duodenum. *FEMS Immunol Med Microbiol* 2003; **36**: 159-168

18 **Quiding-Järbrink M**, Lundin BS, Lönroth H, Svennerholm AM. CD4+ and CD8+ T cell responses in Helicobacter pylori-infected individuals. *Clin Exp Immunol* 2001; **123**: 81-87

19 **Karttunen R**, Andersson G, Poikonen K, Kosunen TU, Karttunen T, Juutinen K, Niemelä S. Helicobacter pylori induces lymphocyte activation in peripheral blood cultures. *Clin Exp Immunol* 1990; **82**: 485-488

20 **Baecher-Allan C**, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* 2001; **167**: 1245-1253

21 **Azem J**, Svennerholm AM, Lundin BS. B cells pulsed with Helicobacter pylori antigen efficiently activate memory CD8+ T cells from H pylori-infected individuals. *Clin Immunol* 2006; **118**: 284-291

22 **D'Elios MM**, Manghetti M, De Carli M, Costa F, Baldari CT, Burroni D, Telford JL, Romagnani S, Del Prete G. T helper 1 effector cells specific for Helicobacter pylori in the gastric antrum of patients with peptic ulcer disease. *J Immunol* 1997; **158**: 962-967

23 **D'Elios MM**, Manghetti M, Almerigogna F, Amedei A, Costa F, Burroni D, Baldari CT, Romagnani S, Telford JL, Del Prete G. Different cytokine profile and antigen-specificity repertoire in Helicobacter pylori-specific T cell clones from the antrum of chronic gastritis patients with or without peptic ulcer. *Eur J Immunol* 1997; **27**: 1751-1755

24 **Kranzer K**, Eckhardt A, Aigner M, Knoll G, Deml L, Speth C, Lehn N, Rehli M, Schneider-Brachert W. Induction of maturation and cytokine release of human dendritic cells by Helicobacter pylori. *Infect Immun* 2004; **72**: 4416-4423

25 **Lenschow DJ**, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 1996; **14**: 233-258

26 **Linsley PS**. Distinct roles for CD28 and cytotoxic T lymphocyte-associated molecule-4 receptors during T cell activation? *J Exp Med* 1995; **182**: 289-292

27 **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034

28 **Suh WK**, Gajewska BU, Okada H, Gronski MA, Bertram EM, Dawicki W, Duncan GS, Bukczynski J, Plyte S, Elia A, Wakeham A, Itie A, Chung S, Da Costa J, Arya S, Horan T, Campbell P, Gaida K, Ohashi PS, Watts TH, Yoshinaga SK, Bray MR, Jordana M, Mak TW. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat Immunol* 2003; **4**: 899-906

29 **Sica GL**, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, Chapoval AI, Flies DB, Bajorath J, Chen L. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* 2003; **18**: 849-861

30 **Chapoval AI**, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, Dong H, Sica GL, Zhu G, Tamada K, Chen L. B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. *Nat Immunol* 2001; **2**: 269-274

31 **Das S**, Suarez G, Beswick EJ, Sierra JC, Graham DY, Reyes VE. Expression of B7-H1 on gastric epithelial cells: its potential role in regulating T cells during Helicobacter pylori infection. *J Immunol* 2006; **176**: 3000-3009

32 **Anderson KM**, Czinn SJ, Redline RW, Blanchard TG. Induction of CTLA-4-mediated anergy contributes to persistent colonization in the murine model of gastric Helicobacter pylori infection. *J Immunol* 2006; **176**: 5306-5313

33 **Sprent J**. Direct stimulation of naïve T cells by antigen-presenting cell vesicles. *Blood Cells Mol Dis* 2005; **35**: 17-20

34 **Raposo G**, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996; **183**: 1161-1172

35 **Zitvogel L**, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G, Amigorena S. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* 1998; **4**: 594-600

36 **Guiney DG**, Hasegawa P, Cole SP. Helicobacter pylori preferentially induces interleukin 12 (IL-12) rather than IL-6 or IL-10 in human dendritic cells. *Infect Immun* 2003; **71**: 4163-4166

37 **Voland P**, Weeks DL, Marcus EA, Prinz C, Sachs G, Scott D. Interactions among the seven Helicobacter pylori proteins encoded by the urease gene cluster. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G96-G106

38 **Appelmelk BJ**, van Die I, van Vliet SJ, Vandenbroucke-Grauls CM, Geijtenbeek TB, van Kooyk Y. Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *J Immunol* 2003; **170**: 1635-1639

39 **Dunn BE**, Vakil NB, Schneider BG, Miller MM, Zitzer JB, Peutz T, Phadnis SH. Localization of Helicobacter pylori urease and heat shock protein in human gastric biopsies. *Infect Immun* 1997; **65**: 1181-1188

40 **Harris PR**, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology* 1996; **111**: 419-425

41 **Ye G**, Barrera C, Fan X, Gourley WK, Crowe SE, Ernst PB, Reyes VE. Expression of B7-1 and B7-2 costimulatory molecules by human gastric epithelial cells: potential role in CD4+ T cell activation during Helicobacter pylori infection. *J Clin Invest* 1997; **99**: 1628-1636

42 **Barrera C**, Ye G, Espejo R, Gunasena S, Almanza R, Leary J, Crowe S, Ernst P, Reyes VE. Expression of cathepsins B, L, S, and D by gastric epithelial cells implicates them as antigen presenting cells in local immune responses. *Hum Immunol* 2001; **62**: 1081-1091

43 **Yoshikawa T**, Naito Y. The role of neutrophils and inflammation in gastric mucosal injury. *Free Radic Res* 2000; **33**: 785-794

44 **Gobert AP**, Mersey BD, Cheng Y, Blumberg DR, Newton JC, Wilson KT. Cutting edge: urease release by Helicobacter pylori stimulates macrophage inducible nitric oxide synthase. *J Immunol* 2002; **168**: 6002-6006

45 **Gobert AP**, McGee DJ, Akhtar M, Mendz GL, Newton JC, Cheng Y, Mobley HL, Wilson KT. Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc Natl Acad Sci USA* 2001; **98**: 13844-13849

46 **Fischer W**, Püls J, Buhrdorf R, Gebert B, Odenbreit S, Haas R. Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. *Mol Microbiol* 2001; **42**: 1337-1348

47 **Mimuro H**, Suzuki T, Tanaka J, Asahi M, Haas R, Sasakawa C. Grb2 is a key mediator of helicobacter pylori CagA protein activities. *Mol Cell* 2002; **10**: 745-755

48 **Beswick EJ**, Pinchuk IV, Suarez G, Sierra JC, Reyes VE. Helicobacter pylori CagA-dependent macrophage migration inhibitory factor produced by gastric epithelial cells binds to CD74 and stimulates procarcinogenic events. *J Immunol* 2006; **176**: 6794-6801

49 **Wang J**, Fan X, Lindholm C, Bennett M, O'Connoll J, Shanahan F, Brooks EG, Reyes VE, Ernst PB. Helicobacter pylori modulates lymphoepithelial cell interactions leading to epithelial cell damage through Fas/Fas ligand interactions. *Infect Immun* 2000; **68**: 4303-4311

50 **Ishihara S**, Rumi MA, Kadowaki Y, Ortega-Cava CF, Yuki T, Yoshino N, Miyaoka Y, Kazumori H, Ishimura N, Amano Y, Kinoshita Y. Essential role of MD-2 in TLR4-dependent signaling during Helicobacter pylori-associated gastritis. *J Immunol* 2004; **173**: 1406-1416

51 **Schmausser B**, Andrulis M, Endrich S, Lee SK, Josenhans C, Müller-Hermelink HK, Eck M. Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection. *Clin Exp Immunol* 2004; **136**: 521-526

52 **Schmausser B**, Andrulis M, Endrich S, Müller-Hermelink HK, Eck M. Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with Helicobacter pylori. *Int J Med Microbiol* 2005; **295**: 179-185

53 **Smith MF Jr**, Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, Goldberg JB. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem* 2003; **278**: 32552-32560

54 **Bliss CM Jr**, Golenbock DT, Keates S, Linevsky JK, Kelly CP. Helicobacter pylori lipopolysaccharide binds to CD14 and stimulates release of interleukin-8, epithelial neutrophil-activating peptide 78, and monocyte chemotactic protein 1 by human monocytes. *Infect Immun* 1998; **66**: 5357-5363

55 **Muotiala A**, Helander IM, Pyhälä L, Kosunen TU, Moran AP. Low biological activity of Helicobacter pylori lipopolysaccharide. *Infect Immun* 1992; **60**: 1714-1716

56 **Cunningham MD**, Seachord C, Ratcliffe K, Bainbridge B, Aruffo A, Darveau RP. Helicobacter pylori and Porphyromonas gingivalis lipopolysaccharides are poorly transferred to recombinant soluble CD14. *Infect Immun* 1996; **64**: 3601-3608

57 **Andersen-Nissen E**, Smith KD, Strobe KL, Barrett SL, Cookson BT, Logan SM, Aderem A. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci USA* 2005; **102**: 9247-9252

58 **Nakajima S**, Krishnan B, Ota H, Segura AM, Hattori T, Graham DY, Genta RM. Mast cell involvement in gastritis with or without Helicobacter pylori infection. *Gastroenterology* 1997; **113**: 746-754

59 **Velin D**, Bachmann D, Bouzourene H, Michetti P. Mast cells are critical mediators of vaccine-induced Helicobacter clearance in the mouse model. *Gastroenterology* 2005; **129**: 142-155

60 **Viala J**, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Mémet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ, Ferrero RL. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* 2004; **5**: 1166-1174

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EDITORIAL

H pylori and host interactions that influence pathogenesis

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Abstract

H pylori is probably the most prevalent human pathogen worldwide. Since it was initially suggested in 1983 by Marshall and Warren to be implicated in gastritis and peptic ulcer disease, *H pylori* has also been implicated in gastric carcinoma and was classified as a class I carcinogen. In the last two decades, a noteworthy body of research has revealed the multiple processes that this gram negative bacterium activates to cause gastroduodenal disease in humans. Most infections are acquired early in life and may persist for the life of the individual. While infected individuals mount an inflammatory response that becomes chronic, along with a detectable adaptive immune response, these responses are ineffective in clearing the infection. *H pylori* has unique features that allow it to reside within the harsh conditions of the gastric environment, and also to evade the host immune response. In this review, we discuss the various virulence factors expressed by this bacterium and how they interact with the host epithelium to influence pathogenesis.

Key words: *H pylori*; Gastric cancer; Immune response; Vacuolating cytotoxin

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H PYLORI INFECTION AND DISEASES ASSOCIATED WITH THE INFECTION

H pylori is one of the most common pathogens affecting humankind, infecting approximately 50% of the world's population. This pathogen is a gram-negative spiral shaped bacterium that has the unique ability to colonize the human gastric mucosa. The infection is usually acquired early in life and may persist a lifetime, unless treated. Of those infected, many will develop asymptomatic gastritis, but 10% develop gastric or duodenal ulcers, and approximately 1% develop gastric carcinoma. The outcome of the infection may involve a combination of the bacterial factors, host factors, as well as environmental factors. Ulceration and carcinogenesis are mutually exclusive outcomes of this infection. *H pylori* infection is a very persistent infection, and in areas of high prevalence, reinfection is also very common.

A very high percentage of gastric and duodenal ulcers (up to 85%) are attributable to *H pylori* infection. Patients in the United States who are infected with *H pylori* have a 3.5 times increased risk of developing peptic ulcer disease than uninfected persons[1]. A hallmark feature of infection with *H pylori* is a pronounced inflammatory response and the inability of the host to clear the infection, which results in a persistent infection, increased acid production, and tissue damage.

It is now well accepted that chronic infection with *H pylori* is a major risk factor in the development of gastric cancer. *H pylori* has been shown to induce changes in the gastric mucosa that could contribute to the development of cancer. Given the strength of the evidence supporting an association between adenocarcinomas of the gastric mucosa and *H pylori* infection, *H pylori* has become classified as a class I carcinogen by the International Agency for Research on Cancer in affiliation with the World Health Organization[2]. Gastric cancer remains the second deadliest cancer worldwide. On a global scale, gastric cancer accounts for approximately 700 000 deaths annually. In the US there are 24 000 new cases and 14 000 deaths annually[3].

Infection with *H pylori* also plays a critical role in the development of mucosa-associated lymphoid tissue (MALT) lymphoma. *H pylori* is present in the gastric mucosa of most cases of MALT lymphoma, and 75% of these cases regress after eradication of *H pylori*[4,5]. Interestingly, gastric MALT lymphoma is the only known

Table 1 *H pylori* **adhesins and the gastric epithelial receptor for each**

Adhesin	Receptor
BabA	Lewis B blood group antigen
SabA/B	Sialyl Lewis X
HpaA	Sialyl Lewis X
UreB	CD74
UreA?	Class Ⅱ MHC
AlpA/B	?
HopZ	?
?	DAF
?	Sulfated molecules (heparan sulfate)
?	Phospholipids
?	Trefoil Factor 1

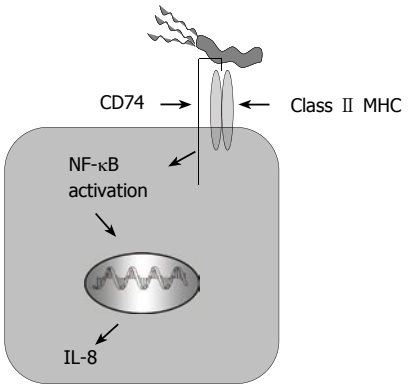

Figure 1 *H pylori* binds to CD74 on gastric epithelial cells and induces NF-κB activation and IL-8 production.

malignancy whose course can be directly changed by the removal of a pathogen. Thus, *H pylori*-associated diseases are a significant global problem and result in considerable morbidity, mortality, and societal costs.

H pylori virulence factors and pathogenesis

H pylori colonizes the gastric epithelial apical surface, but the precise mechanisms of adherence and pathogenesis are still being elucidated. Adherent strains are able to survive in the gastric mucosa, colonize at high densities, and are able to re-colonize, while non-adherent strains are readily removed[6]. Thus, adhesion is crucial in the ability of *H pylori* to persist and cause disease. In addition to contributing to colonization, adherence results in signal transduction, activation of NF-κB and subsequent secretion of interleukin-8, which is important in the inflammatory response during infection.

Adhesins

An assortment of molecules on gastric epithelial cells (GECs) have been proposed as receptors for *H pylori* adherence, as well as multiple adhesins that have been identified on the outer membrane of *H pylori,* but those responsible for pathogenic events are still being investigated (Table 1). Several well known adhesins are BabA, SabA, and AlpAB. BabA and SabA bind to fucosylated and sialylated blood group antigens, respectively**.** There are clearly multiple adhesins and receptors for *H pylori* because only half of the strains in the U.S. have detectable BabA[7]. While the attachment of *H pylori* using BabA as an adhesin does not appear to induce signaling or immune responses from host cells, SabA appears to be required for activation of neutrophils and the resulting oxidative burst by binding to sialylated neutrophil receptors[8]. Although the AlpAB receptor is unknown, it may be even more important as an adhesin because studies with knockout strains dramatically reduced adherence of the bacteria to some cells[9]. HopZ, another adhesin being investigated, also showed decreased adherence when a knockout strain was utilized[10], but not as dramatically as the AlpAB knockout strain. *H pylori* urease can also act as a bacterial adhesin[11]. Urease present on the bacterial surface due to bacterial lysis or release[12,13] binds to class Ⅱ MHC molecules on host cells, and may induce their apoptosis[11].

Other studies from this group also suggest that the urease B subunit binds to CD74, which is expressed in polarized fashion on the luminal side of the epithelium[14], and in doing so stimulates gastric epithelial IL-8 release[15] (Figure 1). A recent study by O'Brien and colleagues showed that, similar to other pathogens, *H pylori* uses decay accelerating factor (DAF aka CD55) as a receptor for binding to the gastric epithelium[16]. Since there have been multiple *H pylori* adhesins described, bacterial adhesion is clearly a complex mechanism with multiple outcomes depending on the host cell receptor engaged.

Cytotoxicity associated pathogenicity island

Following colonization and attachment, various virulence factors expressed by certain *H pylori* strains appear to promote disease. For instance, the expression of *cagA* and *vacA* genes by strains of *H pylori* is highly associated with disease[17]. These *cagA* gene-expressing strains have also been associated with peptic ulcer and patients infected with these strains have an increased risk of gastric cancer[18]. The *cagA* gene is considered a marker for a cluster of genes referred to as pathogenicity island (PAI). *cagPAI* is known to encode for a type IV secretion system that allows CagA, and possibly peptidoglycan, to be delivered into epithelial cells (Figure 2). CagA is tyrosine phosphorylated by Src family kinases[19], and has differing numbers of tyrosine phosphorylation motifs (EPIYA motifs), which determine the virulence of the *H pylori* strain and host cell response to it. The amount of EPIYA motifs is directly related to the levels of phosphorylation and cytoskeletal rearrangement seen in epithelial cells[20]. Phosphorylated CagA interacts with a protein tyrosine phosphatase, SHP-2 inducing its phosphatase activity. Upon activation of SHP-2, it is able to induce host cells signaling, such as MAP kinase/MEK/ERK1/2 signaling through Ras/Raf. Dysregulation in this pathway is responsible for increased cell proliferation and moving of gastric epithelial cells (cell spreading) and cell elongation (hummingbird phenotype)[21]. Interaction of CagA with other signaling molecules such as growth factor receptor-binding protein-2 (Grb-2), hepatocyte growth factor scatter factor receptor (c-Met), and phospholipase C gamma (PLC-γ) can induce similar phenotypes in gastric epithelial cells[22,23]. Phosphorylated CagA inhibits the activity of Src kinases in a negative feedback loop[19]. Thus, the inhibition on Src kinases activity also results in dephosphorylation of a set

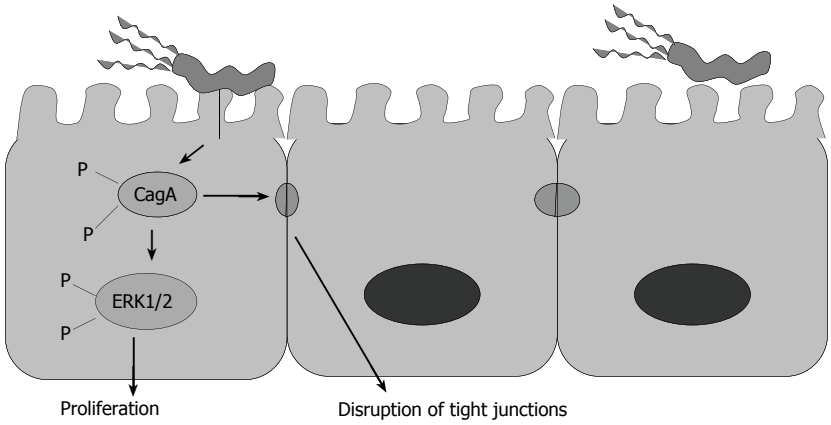

Figure 2 CagA is injected into the cell by a type Ⅳ secretion system where it is phosphorylated and induces ERK1/2 phosphorylation and increased cell proliferation. CagA also induces disruption of the tight junctions between adjacent cells.

of host cell proteins, including the actin-binding protein cortactin. In parallel to ERK1/2 signaling, NF-κB may also be activated, and upregulation of pro-inflammatory cytokines may also ensue[24]. While it was initially thought that the CagA injection was not responsible for the production of proinflammatory cytokines, but other proteins of the *cagPAI* were thought to play a crucial role, it has more recently been shown that CagA injection into the host gastric epithelial cell can induce NF-κB activation and IL-8 production[24]. In fact, independent studies showed that transfection of *cagA* into gastric epithelial cells induced IL-8 production[25]. The activation of the Ras/MEK/ERK pathway occurs following the interaction of CagA with Grb2. Because CagA interacts with important signaling mediators in the host cells, it is considered responsible for changes in cell morphology, adhesion and turnover. CagA also induces the recruitment of ZO-1 and junctional adhesion molecule (JAM) to the site of bacterial attachment causing disruption of the epithelial barrier and dysplastic alterations in epithelial cell morphology. This effect is phosphorylation independent[26]. The delivery of *H pylori* peptidoglycan via the *cagPAI*-encoded type IV secretion system results in the intracellular binding of peptidoglycan by Nod1, an intracellular pathogen-recognition molecule, and this may also contribute to the induction of gastric epithelial responses[27]. The *H pylori* Type IV secretion system (T4SS) stimulates the Rho family GTPases Rac1 and Cdc42 in gastric epithelial cells in an independent CagA translocation mechanism[28]. Thus, Rac1 and Cdc42 are recruited to the membrane at sites of infection activating p21-activated kinase (PAK1) and rearrangements of the cytoskeleton[29]. The T4SS can also activate the receptor tyrosine kinase, epidermal growth factor receptor (EGFR), regulating the ERK1/2 pathway *via* Ras phosphorylation[30]. Furthermore, CagA can inhibit B-cell proliferation by suppressing the JAK-STAT signaling pathway. Hence CagA, in a phosphorylation-independent pathway, can diminish the immune response against *H pylori* and play a role in the development of MALT lymphoma by impairing p53-dependent apoptosis pathway[31].

Vacuolating cytotoxin

The vacuolating toxin, or VacA, is a pore forming toxin that has the ability to induce vacuole formation in cells and disrupt normal membrane trafficking. VacA, is expressed by about half of all *H pylori* strains. Like CagA, VacA appears to be unique to *H pylori* since no other species have a homologue. VacA has effects on many cell types, including gastric epithelial cells, antigen presenting cells, mast cells, and lymphocytes, which makes it an important virulence factor. This toxin is secreted by *H pylori*, and it binds to the plasma membrane of host cells where it forms anion-selective channels. The receptors for VacA are EGFR and RPTP-α and -β[32]. However, VacA also binds to detergent-resistant microdomains (lipid rafts) and GPI-APs[33,34]. This process results in the release of nutrients from the cell, and the bacteria may use these for survival[35]. As the anion concentration becomes higher inside the cell through these pores, proton pumping also increases, as does an influx of weak bases. The weak bases are protonated and trapped inside, causing osmotic swelling, and the formation of a vacuole[36]. VacA can also disrupt mitochondrial membrane potential and affect cellular ATP concentrations, which disrupt the cell cycle progression and lead to apoptosis[37,38]. Another significant way in which VacA contributes to pathogenesis is by inhibiting the processing of antigens by B-cells and their presentation to CD4+ T-cells[39], as well as the T cell activation and proliferation. When mixed with T cells, VacA supresses NFAT[40,41,42], IL-2 production, and surface expression of IL-2 receptors, which are required for T cell proliferation and viability[36]. Multiple signaling pathways of T cell activation are also affected by VacA exposure, which is one mechanism *H pylori* may use to evade immune responses. In gastric epithelial cells, VacA activates the p38 and ERK-1/2 MAP kinases, thereby contributing to the induction of immune responses by these cells[43]. In fact, the *vac*A gene product has been shown to cause in mice, some of the tissue damage observed in *H pylori*-infected patients[44].

Urease

Another major virulence factor of *H pylori* is urease, which is expressed by all strains. Urease is composed of two subunits, α, which is approximately 24 kDa, and β, which is approximately 68 kDa. *H pylori* produces a large amount of urease, representing 5% to 10% of the total protein content of the bacteria. This enzyme is essential for the survival and pathogenesis of the bacteria. Perhaps the most important role urease plays is to hydrolyze urea into CO_2 and NH_3, which aids in buffering the bacteria from

the acidic conditions it may encounter in the stomach. Urease is crucial for *H pylori* colonization, as shown by studies where urease negative strains were not able to colonize in multiple animal studies[45,46]. The inability of urease negative strains to colonize was initially assumed to be due to their inability to buffer their niche. However, similar studies under hypoacidic conditions also led to the same results, where urease negative mutants of *H pylori* could not colonize in an animal model. These observations suggested a role for urease beyond its enzymatic function. Although much urease is located intracellularly, there is some present on the bacterial surface[47,13]. *H pylori* surface-associated urease can act as an adhesin for the bacteria, which induces the production of inflammatory cytokines from both gastric epithelial cells and macrophages[48,49], along with apoptosis of some cells[11]. While the mechanism of action associated with these responses elevated by urease is not entirely clear, the induction of apoptosis may result as a consequence of binding to class Ⅱ MHC[50]. The urease B subunit can also bind to CD74 and induce IL-8 production by gastric epithelial cells[51]. Both of these responses are important in the overall pathogenesis seen during *H pylori* infection.

Other factors

Another important disease-associated virulence factor of *H pylori* is the outer inflammatory protein A (OipA). OipA is part of a family of 32 outer membrane proteins characterized as part of the *H pylori* genome. This protein has been suggested to induce pro-inflammatory responses from gastric epithelial cell lines. In one study with *H pylori* clinical isolates, those isolates expressing OipA, but not the *cag* pathogenicity island proteins were able to induce IL-8 production from gastric epithelial cell lines at 3 times the level of strains that did not express either[52]. Isolates from Japan all expressed OipA, while isolates from the U.S. did not, and thus it is thought that the presence of OipA may make Japanese strains more virulent. When the signaling induced by cagPAI proteins was compared to OipA, OipA was found to induce phosphorylation of Stat1, while the cagPAI proteins induced NF-κB activation[52]. Both of these signaling pathways contribute to induction of IL-8 production, but act in conjunction with one another to fully activate the IL-8 promoter.

Some other virulence factors expressed by *H pylori* include neutrophil-activating protein (NAP) and heat shock protein 60 (Hsp60)[53,54]. NAP has been shown to be a chemoattractant for both monocytes and neutrophils during *H pylori* infection[55]. Hsp60 has been shown to induce proinflammatory cytokines by macrophages and gastric epithelial cells[56,57], which appears to be mediated by Toll-like receptors (TLRs). Lipopolysaccharide expressed by *H pylori* is a very weak immunogen compared to that of other gram negative bacteria, but it has been shown to induce some proinflammatory cytokines[58]. Although the interactions that initiate epithelial cell signals following bacterial adherence are critical in pathogenesis, they are not well understood, nor are the responses of the gastric epithelium that contribute to chronicity of the infection.

In a VacA paralogue mechanism *H pylori* can secrete collagenase, which can degrade collagen present in the extracellular matrix to supply the bacteria with essential amino acids. During chronic infections, collagenase can exacerbate ulcer development and deter the process of ulcer healing[59].

VIRULENCE FACTORS AND INFLAMMATION

Perhaps the most important response for the pathogenesis associated with infection is inflammation. Both antigen specific and non-specific responses contribute to inflammation during infection. These responses contribute to fighting infection, but are also responsible for mucosal damage. Adhesion of *H pylori* to the host epithelium, or bacterial factors such as urease or the cagPAI proteins, induce signaling that upregulates proinflammatory cytokines and chemokines such as IL-8 and GRO-α. In a recent study, we showed that CagA is important in the induction of macrophage migration inhibitory factor (MIF) production by the gastric epithelium[60]. This cytokine has significant importance in the innate and adaptive host responses. Although the cagPAI proteins are the most recognized factor inducing inflammatory responses, there are several other interactions known to upregulate these responses. We have recently discovered that through *H pylori* attachment to CD74, or crosslinking CD74 on gastric epithelial cells, NF-κB activation occurs leading to IL-8 production[15]. Blocking this interaction with monoclonal antibodies resulted in a substantial decrease in the amount of IL-8 produced in response to *H pylori*. Other bacterial factors that induce inflammatory responses are HSP60, which was shown to induce IL-8 through Toll-like receptor pathways[57], and urease, which induced responses from both gastric epithelial cells and peripheral blood mononuclear cells[49]. The cytokine responses, in turn, recruit other immune cells to the site of infection such as IL-8, which is one of the initial chemokines that recruits neutrophils to the site of infection. Neutrophils are then activated by *H pylori* or its soluble products, and proceed to release reactive oxygen species (ROS) and more IL-8[61], which lead to tissue damage associated with infection.

THE IMPACT OF *H PYLORI* ON THE GASTRIC EPITHELIUM

Cell turnover rate

Another response of GECs to infection is enhanced proliferation. In order to balance the increased growth of epithelial cells, the host must compensate by increasing epithelial cell turnover. One mechanism to account for epithelial cell turnover is increased cell death. Apoptosis provides a highly regulated mechanism for cell loss in both healthy and inflamed tissue. In the digestive tract, apoptosis has been described as being important in the control of normal epithelial cell turnover while it is increased in the gastric epithelium during infection with *H pylori*[62,63]. The rate of apoptosis induction may be regulated by exogenous cytokines and growth factors. For example, we have shown that IFN-γ can directly augment the ability of *H pylori* to induce apoptosis of GECs[50]. An indirect effect of IFN-γ is its ability to induce an increased expression of putative receptors (i.e., class Ⅱ MHC and CD74) for *H pylori*. Our

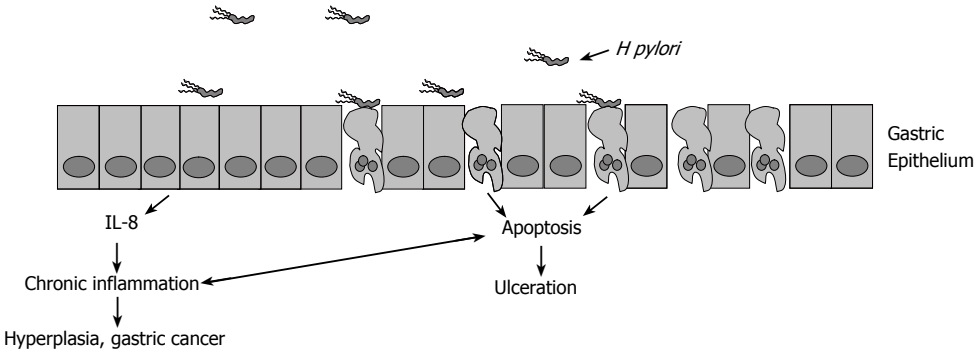

Figure 3 *H pylori* induces chronic inflammation leading to either ulceration or malignant outgrowths.

studies have shown that *H pylori* induces the expression of class Ⅱ MHC and CD74[50,64], and another study showed that *H pylori* induces yet another receptor, sialyl-Le^x, on GEC[65].

Other cytokines and chemokines induced by *H pylori* may also play a role in inducing GEC proliferation. MIF, which is produced by GEC in response to CagA injection[60], and may be produced by macrophages and T cells during infection[66] can induce GEC proliferation. MIF affects proliferation by inactivating p53 tumor suppressor gene and inducing proliferative signaling such as ERK1/2 activation. Additionally, IL-8 has recently been shown to promote GEC proliferation by accelerating the processing of EGFR ligands, which bind and induce transactivation of the receptor[67]. In one study administering nonsteroidal anti-inflammatory drugs was capable of decreasing GEC proliferation in the mouse model[68], further suggesting a role for pro-inflammatory cytokines in cell turnover during *H pylori* infection.

ROS and DNA damage

The prevalence of IL-8 produced by the gastric epithelium at the site of infection results in the infiltration of neutrophils. *H pylori* soluble factors activate neutrophils[69], which go on to produce reactive oxygen species (ROS). Gastric epithelial cells have also been shown to produce ROS in response to *H pylori*[70]. Interestingly, *H pylori* appear to be resistant to the antimicrobial action of ROS. However, ROS may also induce DNA damage in the epithelium, which leads to apoptosis. Since ROS production appears to be dependant on bacterial load[71], it also may be correlated to the amount of damage to the epithelium. When there is a lower bacterial load, the balance between oxidants and antioxidants in the gastric mucosa is disrupted at levels not high enough to induce apoptosis. The risk of DNA damage from ROS is high, and thereby may lead to pro-carcinogenic events.

Overview of the impact of H pylori on pathogenesis

The interactions of *H pylori* with the host are a complex series of events that induce pathogenesis while allowing the bacterium to persist. Only about 20% of the bacteria are bound to the epithelium *via* multiple adhesions at any given time[72]. Attachment to the epithelium, along with multiple virulence factors, induce proinflammatory immune responses. These responses can affect host cell viability and lead to one of two mutually exclusive events. Either excess gastric acid is produced leading to ulceration, or chronic inflammation induces atrophy of the stomach wall and malignant outgrowths (Figure 3). The unique and persistent interactions of *H pylori* with the host, along with 50% worldwide infection rates, make it a significant pathogen that induces considerable disease.

SUMMARY

While a significant volume of knowledge has been acquired during the last decade to help us understand how *H pylori* causes disease, there is still no available vaccine that is effective against this pathogen. *H pylori*'s ability to maintain its long-term residence in a broad segment of humankind is in large part due to the subversion of common structures on the host cells for its interactions. As with most infectious agents, adhesion to host cells is a crucial step in colonization. Attachment is facilitated by various structures or adhesins on the bacteria which include BabA, SabA, HopZ, AlpA/B, and urease. These adhesins bind to carbohydrate moieties on blood group antigens, as is the case of BabA and SabA binding to Lewis antigen, or to proteins of central importance to the host response, such as urease binding to class Ⅱ MHC and CD74. These interactions are important to characterize in detail as they may offer insights into novel prophylactic or therapeutic agents directed at *H pylori*-associated diseases.

Following colonization, *H pylori* employs virulence factors, such as the cagPAI type Ⅳ secretion system and a vacuolating cytotoxin to exert damage on the host epithelium and to alter the host immune response. The ability of VacA to disrupt endosomal traffic and thus alter antigen presentation, together with its ability to arrest T cell cycle progression, makes VacA an important virulence factor that could contribute to the establishment of chronic infection. As we understand the mechanisms that contribute to the long term residence of *H pylori* in the human stomach, we will be better able to prevent the chronicity that underlies the development of the serious diseases associated with this infection.

REFERENCES

1 **Nomura A**, Stemmermann GN, Chyou PH, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* infection and the risk for duodenal and gastric ulceration. *Ann Intern Med* 1994; **120**: 977-981
2 Infection with *Helicobacter pylori*. *IARC Monogr Eval Carcinog Risks Hum* 1994; **61**: 177-240
3 Cancer Database. Available from: http://www-dep iarc fr/2005
4 **Nakamura S**, Yao T, Aoyagi K, Iida M, Fujishima M,

Tsuneyoshi M. *Helicobacter pylori* and primary gastric lymphoma. A histopathologic and immunohistochemical analysis of 237 patients. *Cancer* 1997; **79**: 3-11
5 **Parsonnet J**, Isaacson PG. Bacterial infection and MALT lymphoma. *N Engl J Med* 2004; **350**: 213-215
6 **Hayashi S**, Sugiyama T, Asaka M, Yokota K, Oguma K, Hirai Y. Modification of *Helicobacter pylori* adhesion to human gastric epithelial cells by antiadhesion agents. *Dig Dis Sci* 1998; **43**: 56S-60S
7 **Hennig EE**, Mernaugh R, Edl J, Cao P, Cover TL. Heterogeneity among *Helicobacter pylori* strains in expression of the outer membrane protein BabA. *Infect Immun* 2004; **72**: 3429-3435
8 **Unemo M**, Aspholm-Hurtig M, Ilver D, Bergström J, Borén T, Danielsson D, Teneberg S. The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for nonopsonic activation of human neutrophils. *J Biol Chem* 2005; **280**: 15390-15397
9 **Odenbreit S**, Faller G, Haas R. Role of the alpAB proteins and lipopolysaccharide in adhesion of *Helicobacter pylori* to human gastric tissue. *Int J Med Microbiol* 2002; **292**: 247-256
10 **Peck B**, Ortkamp M, Diehl KD, Hundt E, Knapp B. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. *Nucleic Acids Res* 1999; **27**: 3325-3333
11 **Fan X**, Gunasena H, Cheng Z, Espejo R, Crowe SE, Ernst PB, Reyes VE. *Helicobacter pylori* urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. *J Immunol* 2000; **165**: 1918-1924
12 **Bode G**, Malfertheiner P, Lehnhardt G, Nilius M, Ditschuneit H. Ultrastructural localization of urease of *Helicobacter pylori*. *Med Microbiol Immunol* 1993; **182**: 233-242
13 **Rokita E**, Makristathis A, Hirschl AM, Rotter ML. Purification of surface-associated urease from *Helicobacter pylori*. *J Chromatogr B Biomed Sci Appl* 2000; **737**: 203-212
14 **Barrera CA**, Beswick EJ, Sierra JC, Bland D, Espejo R, Mifflin R, Adegboyega P, Crowe SE, Ernst PB, Reyes VE. Polarized expression of CD74 by gastric epithelial cells. *J Histochem Cytochem* 2005; **53**: 1481-1489
15 **Beswick EJ**, Bland DA, Suarez G, Barrera CA, Fan X, Reyes VE. *Helicobacter pylori* binds to CD74 on gastric epithelial cells and stimulates interleukin-8 production. *Infect Immun* 2005; **73**: 2736-2743
16 **O'Brien DP**, Israel DA, Krishna U, Romero-Gallo J, Nedrud J, Medof ME, Lin F, Redline R, Lublin DM, Nowicki BJ, Franco AT, Ogden S, Williams AD, Polk DB, Peek RM Jr. The role of decay-accelerating factor as a receptor for *Helicobacter pylori* and a mediator of gastric inflammation. *J Biol Chem* 2006; **281**: 13317-13323
17 **Leunk RD**. Production of a cytotoxin by *Helicobacter pylori*. *Rev Infect Dis* 1991; **13** Suppl 8: S686-S689
18 **Tee W**, Lambert JR, Dwyer B. Cytotoxin production by *Helicobacter pylori* from patients with upper gastrointestinal tract diseases. *J Clin Microbiol* 1995; **33**: 1203-1205
19 **Selbach M**, Moese S, Hurwitz R, Hauck CR, Meyer TF, Backert S. The *Helicobacter pylori* CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. *EMBO J* 2003; **22**: 515-528
20 **Figueiredo C**, Machado JC, Yamaoka Y. Pathogenesis of *Helicobacter pylori* Infection. *Helicobacter* 2005; **10** Suppl 1: 14-20
21 **Higashi H**, Nakaya A, Tsutsumi R, Yokoyama K, Fujii Y, Ishikawa S, Higuchi M, Takahashi A, Kurashima Y, Teishikata Y, Tanaka S, Azuma T, Hatakeyama M. *Helicobacter pylori* CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *J Biol Chem* 2004; **279**: 17205-17216
22 **Churin Y**, Al-Ghoul L, Kepp O, Meyer TF, Birchmeier W, Naumann M. *Helicobacter pylori* CagA protein targets the c-Met receptor and enhances the motogenic response. *J Cell Biol* 2003; **161**: 249-255
23 **Mimuro H**, Suzuki T, Tanaka J, Asahi M, Haas R, Sasakawa C. Grb2 is a key mediator of helicobacter pylori CagA protein activities. *Mol Cell* 2002; **10**: 745-755
24 **Brandt S**, Kwok T, Hartig R, König W, Backert S. NF-kappaB activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. *Proc Natl Acad Sci USA* 2005; **102**: 9300-5930
25 **Kim SY**, Lee YC, Kim HK, Blaser MJ. *Helicobacter pylori* CagA transfection of gastric epithelial cells induces interleukin-8. *Cell Microbiol* 2006; **8**: 97-106
26 **Amieva MR**, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* 2003; **300**: 1430-1434
27 **Viala J**, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Mémet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philpott DJ, Ferrero RL. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol* 2004; **5**: 1166-1174
28 **Churin Y**, Kardalinou E, Meyer TF, Naumann M. Pathogenicity island-dependent activation of Rho GTPases Rac1 and Cdc42 in *Helicobacter pylori* infection. *Mol Microbiol* 2001; **40**: 815-823
29 **Guillemin K**, Salama NR, Tompkins LS, Falkow S. Cag pathogenicity island-specific responses of gastric epithelial cells to *Helicobacter pylori* infection. *Proc Natl Acad Sci USA* 2002; **99**: 15136-15141
30 **Keates S**, Sougioultzis S, Keates AC, Zhao D, Peek RM Jr, Shaw LM, Kelly CP. cag+ *Helicobacter pylori* induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells. *J Biol Chem* 2001; **276**: 48127-48134
31 **Umehara S**, Higashi H, Ohnishi N, Asaka M, Hatakeyama M. Effects of *Helicobacter pylori* CagA protein on the growth and survival of B lymphocytes, the origin of MALT lymphoma. *Oncogene* 2003; **22**: 8337-8342
32 **Fujikawa A**, Shirasaka D, Yamamoto S, Ota H, Yahiro K, Fukada M, Shintani T, Wada A, Aoyama N, Hirayama T, Fukamachi H, Noda M. Mice deficient in protein tyrosine phosphatase receptor type Z are resistant to gastric ulcer induction by VacA of Helicobacter pylori. *Nat Genet* 2003; **33**: 375-381
33 **Kuo CH**, Wang WC. Binding and internalization of *Helicobacter pylori* VacA via cellular lipid rafts in epithelial cells. *Biochem Biophys Res Commun* 2003; **303**: 640-644
34 **Schraw W**, Li Y, McClain MS, van der Goot FG, Cover TL. Association of *Helicobacter pylori* vacuolating toxin (VacA) with lipid rafts. *J Biol Chem* 2002; **277**: 34642-34650
35 **Szabò I**, Brutsche S, Tombola F, Moschioni M, Satin B, Telford JL, Rappuoli R, Montecucco C, Papini E, Zoratti M. Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity. *EMBO J* 1999; **18**: 5517-5527
36 **Cover TL**, Blanke SR. *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. *Nat Rev Microbiol* 2005; **3**: 320-332
37 **Cover TL**, Krishna US, Israel DA, Peek RM Jr. Induction of gastric epithelial cell apoptosis by *Helicobacter pylori* vacuolating cytotoxin. *Cancer Res* 2003; **63**: 951-957
38 **Kimura M**, Goto S, Wada A, Yahiro K, Niidome T, Hatakeyama T, Aoyagi H, Hirayama T, Kondo T. Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in human gastric cells. *Microb Pathog* 1999; **26**: 45-52
39 **Molinari M**, Salio M, Galli C, Norais N, Rappuoli R, Lanzavecchia A, Montecucco C. Selective inhibition of Ii-dependent antigen presentation by *Helicobacter pylori* toxin VacA. *J Exp Med* 1998; **187**: 135-140
40 **Boncristiano M**, Paccani SR, Barone S, Ulivieri C, Patrussi L, Ilver D, Amedei A, D'Elios MM, Telford JL, Baldari CT. The *Helicobacter pylori* vacuolating toxin inhibits T cell activation by two independent mechanisms. *J Exp Med* 2003; **198**: 1887-1897
41 **Sundrud MS**, Torres VJ, Unutmaz D, Cover TL. Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci USA* 2004; **101**: 7727-7732
42 **Gebert B**, Fischer W, Weiss E, Hoffmann R, Haas R. *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 2003; **301**: 1099-1102
43 **Nakayama M**, Kimura M, Wada A, Yahiro K, Ogushi K,

Niidome T, Fujikawa A, Shirasaka D, Aoyama N, Kurazono H, Noda M, Moss J, Hirayama T. *Helicobacter pylori* VacA activates the p38/activating transcription factor 2-mediated signal pathway in AZ-521 cells. *J Biol Chem* 2004; **279**: 7024-7028

44 **Telford JL**, Ghiara P, Dell'Orco M, Comanducci M, Burroni D, Bugnoli M, Tecce MF, Censini S, Covacci A, Xiang Z. Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J Exp Med* 1994; **179**: 1653-1658

45 **Karita M**, Tsuda M, Nakazawa T. Essential role of urease in vitro and in vivo *Helicobacter pylori* colonization study using a wild-type and isogenic urease mutant strain. *J Clin Gastroenterol* 1995; **21** Suppl 1: S160-S163

46 **Tsuda M**, Karita M, Morshed MG, Okita K, Nakazawa T. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infect Immun* 1994; **62**: 3586-3589

47 **Phadnis SH**, Parlow MH, Levy M, Ilver D, Caulkins CM, Connors JB, Dunn BE. Surface localization of *Helicobacter pylori* urease and a heat shock protein homolog requires bacterial autolysis. *Infect Immun* 1996; **64**: 905-912

48 **Harris PR**, Ernst PB, Kawabata S, Kiyono H, Graham MF, Smith PD. Recombinant *Helicobacter pylori* urease activates primary mucosal macrophages. *J Infect Dis* 1998; **178**: 1516-1520

49 **Takahashi T**, Yujiri T, Shinohara K, Inoue Y, Sato Y, Fujii Y, Okubo M, Zaitsu Y, Ariyoshi K, Nakamura Y, Nawata R, Oka Y, Shirai M, Tanizawa Y. Molecular mimicry by *Helicobacter pylori* CagA protein may be involved in the pathogenesis of H. pylori-associated chronic idiopathic thrombocytopenic purpura. *Br J Haematol* 2004; **124**: 91-96

50 **Fan X**, Crowe SE, Behar S, Gunasena H, Ye G, Haeberle H, Van Houten N, Gourley WK, Ernst PB, Reyes VE. The effect of class II major histocompatibility complex expression on adherence of *Helicobacter pylori* and induction of apoptosis in gastric epithelial cells: a mechanism for T helper cell type 1-mediated damage. *J Exp Med* 1998; **187**: 1659-1669

51 **Beswick EJ**, Pinchuk IV, Minch K, Suarez G, Sierra JC, Yamaoka Y, Reyes VE. The *Helicobacter pylori* urease B subunit binds to CD74 on gastric epithelial cells and induces NF-kappaB activation and interleukin-8 production. *Infect Immun* 2006; **74**: 1148-1155

52 **Yamaoka Y**, Kwon DH, Graham DY. A M(r) 34,000 proinflammatory outer membrane protein (oipA) of *Helicobacter pylori*. *Proc Natl Acad Sci USA* 2000; **97**: 7533-7538

53 **Bai Y**, Li LR, Wang JD, Chen Y, Jin JF, Zhang ZS, Zhou DY, Zhang YL. Expression of *Helicobacter pylori* Hsp60 protein and its immunogenicity. *World J Gastroenterol* 2003; **9**: 2711-2714

54 **Yamaguchi H**, Osaki T, Taguchi H, Hanawa T, Yamamoto T, Kamiya S. Relationship between expression of HSP60, urease activity, production of vacuolating toxin, and adherence activity of *Helicobacter pylori*. *J Gastroenterol* 1998; **33** Suppl 10: 6-9

55 **Satin B**, Del Giudice G, Della Bianca V, Dusi S, Laudanna C, Tonello F, Kelleher D, Rappuoli R, Montecucco C, Rossi F. The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is a protective antigen and a major virulence factor. *J Exp Med* 2000; **191**: 1467-1476

56 **Gobert AP**, Bambou JC, Werts C, Balloy V, Chignard M, Moran AP, Ferrero RL. *Helicobacter pylori* heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *J Biol Chem* 2004; **279**: 245-250

57 **Takenaka R**, Yokota K, Ayada K, Mizuno M, Zhao Y, Fujinami Y, Lin SN, Toyokawa T, Okada H, Shiratori Y, Oguma K. *Helicobacter pylori* heat-shock protein 60 induces inflammatory responses through the Toll-like receptor-triggered pathway in cultured human gastric epithelial cells. *Microbiology* 2004; **150**: 3913-3922

58 **Bliss CM Jr**, Golenbock DT, Keates S, Linevsky JK, Kelly CP. *Helicobacter pylori* lipopolysaccharide binds to CD14 and stimulates release of interleukin-8, epithelial neutrophil-activating peptide 78, and monocyte chemotactic protein 1 by human monocytes. *Infect Immun* 1998; **66**: 5357-5363

59 **Kavermann H**, Burns BP, Angermuller K, Odenbreit S, Fischer W, Melchers K, Haas R. Identification and characterization of *Helicobacter pylori* genes essential for gastric colonization. *J Exp Med* 2003; **197**: 813-822

60 **Beswick EJ**, Pinchuk IV, Suarez G, Sierra JC, Reyes VE. *Helicobacter pylori* CagA-dependent macrophage migration inhibitory factor produced by gastric epithelial cells binds to CD74 and stimulates procarcinogenic events. *J Immunol* 2006; **176**: 6794-6801

61 **Shimoyama T**, Fukuda S, Liu Q, Nakaji S, Fukuda Y, Sugawara K. *Helicobacter pylori* water soluble surface proteins prime human neutrophils for enhanced production of reactive oxygen species and stimulate chemokine production. *J Clin Pathol* 2003; **56**: 348-351

62 **Moss SF**, Calam J, Agarwal B, Wang S, Holt PR. Induction of gastric epithelial apoptosis by *Helicobacter pylori*. *Gut* 1996; **38**: 498-501

63 **Mannick EE**, Bravo LE, Zarama G, Realpe JL, Zhang XJ, Ruiz B, Fontham ET, Mera R, Miller MJ, Correa P. Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants. *Cancer Res* 1996; **56**: 3238-3243

64 **Beswick EJ**, Das S, Pinchuk IV, Adegboyega P, Suarez G, Yamaoka Y, Reyes VE. *Helicobacter pylori*-induced IL-8 production by gastric epithelial cells up-regulates CD74 expression. *J Immunol* 2005; **175**: 171-176

65 **Mahdavi J**, Sondén B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadström T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarström L, Borén T. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. *Science* 2002; **297**: 573-578

66 **He XX**, Yang J, Zheng XL, Ding YW, Shen QY, Liu W, Zhao YH. The effect of *Helicobacter pylori* infection on expression of macrophage migration inhibitory factor by T cells and macrophages in gastric mucosa. *Chin Med J* (Engl) 2005; **118**: 1201-1205

67 **Joh T**, Kataoka H, Tanida S, Watanabe K, Ohshima T, Sasaki M, Nakao H, Ohhara H, Higashiyama S, Itoh M. *Helicobacter pylori*-stimulated interleukin-8 (IL-8) promotes cell proliferation through transactivation of epidermal growth factor receptor (EGFR) by disintegrin and metalloproteinase (ADAM) activation. *Dig Dis Sci* 2005; **50**: 2081-2089

68 **Kim TI**, Lee YC, Lee KH, Han JH, Chon CY, Moon YM, Kang JK, Park IS. Effects of nonsteroidal anti-inflammatory drugs on *Helicobacter pylori*-infected gastric mucosae of mice: apoptosis, cell proliferation, and inflammatory activity. *Infect Immun* 2001; **69**: 5056-5063

69 **Suzuki H**, Miura S, Imaeda H, Suzuki M, Han JY, Mori M, Fukumura D, Tsuchiya M, Ishii H. Enhanced levels of chemiluminescence and platelet activating factor in urease-positive gastric ulcers. *Free Radic Biol Med* 1996; **20**: 449-454

70 **Bagchi D**, Bhattacharya G, Stohs SJ. Production of reactive oxygen species by gastric cells in association with *Helicobacter pylori*. *Free Radic Res* 1996; **24**: 439-450

71 **Davies GR**, Banatvala N, Collins CE, Sheaff MT, Abdi Y, Clements L, Rampton DS. Relationship between infective load of *Helicobacter pylori* and reactive oxygen metabolite production in antral mucosa. *Scand J Gastroenterol* 1994; **29**: 419-424

72 **Hessey SJ**, Spencer J, Wyatt JI, Sobala G, Rathbone BJ, Axon AT, Dixon MF. Bacterial adhesion and disease activity in Helicobacter associated chronic gastritis. *Gut* 1990; **31**: 134-138

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EDITORIAL

Interleukin-12 and Th1 immune response in Crohn's disease: Pathogenetic relevance and therapeutic inplication

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders of the gastrointestinal tract that share clinical and pathological characteristics. The most accredited hypothesis is that both CD and UC result from a deregulated mucosal immune response to normal constituents of the gut microflora. Evidence, however, indicates that the main pathological processes in these two diseases are distinct. In CD, the tissue-damaging inflammatory reaction is driven by activated type 1 helper T-cell (Th1), whereas a humoral response predominates in UC. Consistently, a marked accumulation of macrophages making interleukin (IL)-12, the major Th1-inducing factor, is seen in CD but not in UC mucosa. Preliminary studies also indicate that administration of a monoclonal antibody blocking the IL-12/p40 subunit can be useful to induce and maintain clinical remission in CD patients. Notably, the recently described IL-23 shares the p40 subunit with IL-12, raising the possibility that the clinical benefit of the anti-IL-12/p40 antibody in CD may also be due to the neutralization of IL-23 activity. This review summarizes the current information on the expression and functional role of IL-12 and IL-12-associated signaling pathways both in patients with CD and experimental models of colitis, thus emphasizing major differences between IL-12 and IL-23 activity on the development of intestinal inflammation.

Key words: Interleukin-12; Type 1 helper T-cell cytokines; Inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD) is the general term indicating Crohn's disease (CD) and ulcerative colitis (UC), two chronic inflammatory disorders of the intestine that have different morphological, immunological and clinical characteristics. The etiology of IBD is unknown, but evidence has been accumulated to show that the liability to develop CD or UC is influenced by a wide range of genetic and environmental factors, which have been only in part characterized. Over the last recent years, it has also become evident that both CD and UC are caused by excessive immune reactivity in the gut wall, and that this is directed against normal constituents of the luminal flora. However, CD and UC are immunologically different diseases, even though they share end-stage effector pathways of tissue damage. These advances led to the development of novel therapeutic agents that are currently being studied for their capacity to specifically target the mucosal inflammatory pathways occurring in IBD patients.

IL-12 AND TH1 CYTOKINES IN CD

In both CD and UC, the inflamed tissue is heavily infiltrated with leukocytes, mostly T lymphocytes. These cells are activated and make increased amounts of cytokines, which are thought to have a primary role in promoting the disease process. Using sensitive assays, several authors have shown that CD and UC have distinct profiles of cytokine production. While in CD there is a predominant synthesis of type 1 helper T-cell (Th1) cytokines, including IFN-γ and TNF-α, Th2 cytokines, such as IL-5 and IL-13, are considered to have a more prominent role in UC[1,2]. T-lamina propria lymphocytes (T-LPL) isolated from the inflamed colon of UC patients also make more IFN-γ than normal T-LPL following *in vitro* activation with anti-CD3/CD28 antibodies[1]. Therefore, the classic Th1-Th2 paradigm seems to be overly simplistic, and there is now sufficient evidence to believe that these two pathways can co-exist rather than being mutually exclusive in the human gut.

The discovery that IFN-γ-secreting T-LPL are abundant in CD mucosa has paved the way for studies in which the switch that controls the differentiation of such cell type was investigated. This research led to the demonstration that in CD mucosa there is increased production of IL-12, the major Th1-inducing factor in man[3,4]. IL-12 is a heterodimeric cytokine composed

of two covalently linked subunits (p40 and p35) and synthesized by monocytes/macrophages/dendritic cells[5]. Transcripts for both IL-12 subunits have been detected in gastric and intestinal mucosa of patients with CD[3,6]. In addition, it was shown that lamina propria mononuclear cells isolated from intestinal mucosal areas of CD, but not UC, patients released *in vitro* functionally active IL-12, and that neutralization of endogenous IL-12, in CD mucosal cell cultures, resulted in a significant decrease in the number of IFN-γ-producing cells[3,4].

IL-12 mediates its biological activities through a receptor composed of two subunits, β1 and β2[5]. Although both subunits are required to form a functional receptor, β2 appears to be crucial in controlling Th1 cell lineage commitment[7,8]. Consistently, high expression of IL-12Rβ2 has been described in various Th1-mediated diseases, as well as in CD T-lamina propria lymphocytes (T-LPL)[9-11]. Additionally, CD mucosal lymphocytes express high levels of active STAT-4, a transcription factor that is activated by IL-12R signals and is necessary to promote the induction of IL-12-driven Th1-associated genes[11]. Notably, T cells from STAT-4-deficient mice manifest impaired IFN-γ production in response to IL-12 and are unable to efficiently promote the development of colitis when transferred in immunodeficient mice[12]. On the other hand, studies in mice over-expressing STAT-4 revealed that such animals developed colitis that is characterized by the presence of a diffuse infiltration of Th1 cytokine-secreting cells in the intestinal wall[13].

While IL-12 appears to be sufficient to trigger the Th1 cell program in naïve T cells, the expansion and maintenance of Th1 cell response in the gut would require additional signals (Figure 1). Indeed, the IL-12-induced synthesis of IFN-γ by intestinal lamina propria T lymphocytes can be enhanced by cytokines that signal through the common γ-chain receptor, such as IL-7, IL-15 and IL-21[14,15]. Additionally, in CD mucosa, there is an enhanced production of biologically active IL-18, a cytokine involved in perpetuating Th1 cell responses[16,17]. Immunohistochemical analysis has localized IL-18 to both lamina propria mononuclear cells and intestinal epithelial cells. In these cells, the expression of IL-18 is invariably associated with active subunits of IL-1β-converting enzyme, a molecule capable of cleaving the precursor form of IL-18 to the active protein[16,17]. Moreover, functional studies showed that down-regulation of IL-18 expression in cultures of CD lamina propria mononuclear cells by specific IL-18 antisense oligonucleotides significantly inhibited IFN-γ synthesis, further supporting the concept that IL-18 serves as a strong costimulatory factor of IL-12-driven Th1 responses[16]. A newly discovered TNF-superfamily cytokine (TL1A) has also been involved in initiating or promoting the Th1 response in CD as well as in experimental models of IBD[18,19]. Another protein that could contribute to the ongoing Th1 immune response in CD is osteopontin, a 60 kDa phosphoprotein, that is highly expressed in epithelial cells and macrophages in CD and shown to increase IL-12 production in CD mucosal cells[20].

An analysis of transcription factors expressed in Th1 *vs* Th2 cells led to the discovery of T-bet, a novel

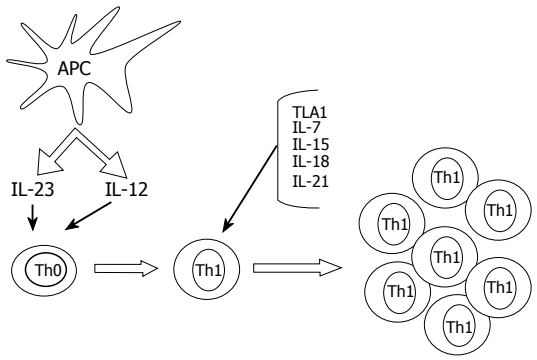

Figure 1 Some putative mechanisms implicated in the induction and expansion of Th1 cells in the gut of patients with Crohn's disease. Cytokines produced by antigen presenting cells, such as IL-23 and IL-12, promote the differentiation of Th1 cells. The expansion and mucosal accumulation of this cell subtype are then sustained by additional molecules, such as IL-18, IL-7, IL-15, IL-21 and TL1A.

member of the T-box family of transcription factors. T-bet drives chromatin remodelling of the IFN-γ locus and up-regulates IL-12Rβ2 chain. Therefore, its expression strictly correlates with the differentiation of Th1 cells[21]. As expected, T-bet is markedly over-expressed in CD4+ T-LPL of patients with CD and it associates with a reduced expression of GATA-3, a transcription factor that governs Th2 cell polarization[22].

The molecular mechanisms underlying T-bet induction are not fully understood, even though there is evidence that cytokines that activate STAT1, such as IFN-γ and IL-21, may positively regulate T-bet expression. In line with these observations, neutralization of IL-21 in cultures of CD mucosal T cells is followed by a decreased expression of T-bet and secretion of IFN-γ[15].

REGULATION OF IL-12 PRODUCTION

A critical question remains as to what induces IL-12 in CD gut and which mechanisms are in place to regulate IL-12 production. IL-12 is produced by antigen-presenting cells mostly in response to bacteria or bacterial products/components[5]. Since the development of Th1-mediated colitis both in humans and mice requires the presence of gut microbiota, it is conceivable that IL-12 production is driven by luminal bacteria. Indeed, it was shown that LPMC isolated from the inflamed intestine of CD patients are hyper-responsive to sonicates of bacteria from autologous intestine (BsA), and this phenomenon associates with increased expression of activation markers on both CD4+ and CD8+ lymphocyte subsets and production of IL-12 and IFN-γ[23]. Consistently, both local and systemic tolerance to BsA is broken in a murine model of chronic intestinal inflammation induced by the hapten reagent 2, 4, 6-trinitrobenzene sulfonic acid (TNBS), which mimics several important characteristics of CD. Tolerance to BsA is, however, restored in mice systemically treated with antibodies to IL-12[24].

The reason why CD LPMC would respond to luminal bacteria with enhanced production of IL-12 remains, however, unclear. One possibility is that, in CD, LPMC are primed to synthesize high levels of IL-12 by specific stimuli. This hypothesis is suggested by the recent

observation that flagellin, a major antigenic target of immune response associated with CD[25], can activate innate immunity *via* Toll-like receptor 5 (TLR5), and instruct dendritic cells to promote Th1 responses *via* IL-12p70 production[26]. Another possibility is that CD LPMC lack negative regulators of bacteria-driven intracellular signals, and therefore would respond to bacterial stimulation with enhanced IL-12 synthesis. In line with this, it has recently been shown that splenocytes of mice carrying on deletions of CARD15, a gene whose mutations are associated with CD, and encoding NOD2, respond to peptidoglycan (PGN) stimulation with exaggerated activation of NF-κB and production of IL-12 and IL-18[27]. According to these data, NOD2 would function as a negative regulator of IL-12 production mediated by PGN. Therefore, in the absence of this negative regulation, PGN could elicit an excessive NF-κB-dependent IL-12 response by mucosal cells[28]. In the gut, NOD2 also regulates the production of anti-bacterial peptides, such as defensin-5, by Paneth cells[29]. Consistently, epithelial cells expressing mutated NOD2 have a reduced capacity to restrict proliferation of bacterial pathogens in monolayer cultures[30], raising the possibility that CARD15 mutations could facilitate the colonization of the intestine with bacteria that eventually sustain macrophage/dendritic cell activation and enhance IL-12-driven Th1 cell responses. Whether this explanation really fits with the mucosal IL-12 synthesis in CD patients with mutations remains, however, unknown, as no study has yet analyzed whether intestinal mucosal cells of CD patients with CARD15 mutations make *in vivo* more IL-12 than those of CD patients without CARD15 mutations.

IL-12-INDUCED T CELL RESPONSE LEADS TO MUCOSAL DESTRUCTION IN HUMAN FETAL GUT

Taken together, the above data suggest that the IL-12-driven Th1 signaling pathway can be important in immune-mediated injury in the gut. This is also substantiated by observations made in *ex vivo* models of T cell-mediated gut inflammation. By using human fetal gut explants, we previously showed that activation of T-LPL by anti-CD3 and IL-12 resulted in a strongly Th1-biased response that was followed by severe tissue injury, with destruction of the mucosa. Furthermore, analysis of explants culture supernatants revealed that stimulation of fetal gut tissues with anti-CD3 and IL-12 increased the production of matrix metalloproteinase 1 (MMP-1, collagenase) and MMP-3 (stromelysin 1), while the synthesis of tissue inhibitors of MMP-1 and 2 remained unchanged[31]. Stromelysin 1 has a broad substrate specificity, being capable of degrading proteoglycans, laminin, fibronectin, collagen core protein, and non-helical cross-linked regions of type IV collagen. Stromelysin 1 has, therefore, the potential to destroy the structure of the intestinal lamina propria, thereby removing the scaffolding on which the epithelium lies. Indeed, abundant stromelysin 1 has been found in the mucosa of patients with CD, particularly near ulcers[32]. Notably, the addition of a stromelysin 1 inhibitor to the IL-12-stimulated fetal gut organ culture prevented the tissue damage without altering T cell activation. Similarly, a p55 TNF receptor human IgG fusion protein was able to prevent the mucosal degradation and inhibit stromelysin 1 production, thus suggesting that TNF-α is a key mediator of the IL-12-induced tissue damage[31].

BLOCKADE OF IL-12 FACILITATES THE RESOLUTION OF TH1-MEDIATED INFLAMMATION IN THE GUT

The role of IL-12 in the mucosal inflammation in patients with CD is also supported by the demonstration that this cytokine is produced in excess in experimental models of Th1-induced colitis, such as the TNBS-induced colitis. Importantly, treatment of mice with antibodies to IL-12/p40 abrogates the TNBS-induced colitis, and the beneficial effect of such a treatment has been linked to the capacity of the blocking antibody to enhance mucosal T cell apoptosis through a FAS-dependent mechanism[33,34].

Consistently, a randomized controlled study of 79 CD patients receiving 1 or 3 mg of an anti-IL-12p40 monoclonal antibody *versus* placebo demonstrated a response in 75% of CD patients compared with 25% in the placebo group. These responses paralleled a decrease in downstream cytokines, including IFN-γ and TNF-α[35]. In subsequent studies, it was also shown that patients with CD manifested both increased IL-12p70 and IL-23 secretion before anti-IL-12p40 mAb treatment and normal levels of secretion of these cytokines following cessation of treatment. Moreover, IL-23-induced T cell production of IL-17 and IL-6 was greatly reduced after IL-12 antibody treatment[36]. More recently, it has been shown that treatment of active CD patients with two doses of Fontolizumab, an anti-IFN-γ antibody, which interferes with Th1 polarization as well as activation of macrophages, monocytes and natural killer cells, resulted in increased rates of clinical response and induction of remission compared with placebo[37].

THE EMERGING ROLE OF IL-23 IN GUT INFLAMMATION

Whereas the central role of IL-12 in the generation of IFN-γ-secreting cells has long been appreciated, recent studies have shown that Th1 cell responses can also be regulated by IL-23. Importantly, IL-23 shares with IL-12 the p40 subunit[38], and therefore, IL-23 biological activity is fully inhibitable by neutralising IL-12p40 antibodies. This fact and the demonstration that IL-23 is up-regulated in CD mucosa[39] raise the question whether the beneficial effect of the blocking IL-12p40 antibody observed in CD patients is due to the neutralization of IL-12 and/or IL-23. Results from studies in mice with targeted deletion of either the IL-12/p35 or IL-23/p19 subunit suggest the possibility that IL-23 and not IL-12 is essential for manifestation of intestinal inflammation occurring in IL-10-deficient mice. The IL-23-driven intestinal inflammation appears to be mediated by the production of IL-17 and IL-6. Moreover, administration of recombinant IL-23 acceler-

ates the development of colitis in lymphocyte-deficient recombinase-activating genes-knockout (RAG-KO) mice after reconstitution with CD4+ T cells from interleukin-10-knockout (IL-10-KO) mice[40]. Whether IL-23 plays a similar role in other models of Th1-mediated colitis, such as the TNBS-colitis, remains however unknown. Similarly, it remains to be ascertained whether the deleterious effect of IL-23 on the ongoing mucosal inflammation occurs only in the absence of IL-10-related regulatory effects.

In conclusion, human IBD are thought to be caused by a dysregulated T cell response directed against constituents of the intestinal bacterial microflora. In CD, such a response is associated with an exaggerated production of IL-12 and IFN-γ. There is also evidence that the recently described IL-23 may drive the intestinal inflammation in murine models of IBD, thus suggesting that strategies aimed at specifically inhibiting the p19 subunit of IL-23 could be therapeutically useful in CD. Some observations made in cell systems, however, suggest to be cautious before drawing any conclusion. In fact, it has been reported that T-bet negatively regulates IL-17 production, thus promoting the shift of IL-17-producing cells towards a classical Th1 phenotype characterized by high IFN-γ[41]. Based on these findings, it is conceivable that IL-23 may play a determinant role in the early phase of T cell-mediated immune responses, thus leaving the place to IL-12/IFN-γ/T-bet pathway in the late phase.

REFERENCES

1 **Fuss IJ**, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 1996; **157**: 1261-1270

2 **Heller F**, Florian P, Bojarski C, Richter J, Christ M, Hillenbrand B, Mankertz J, Gitter AH, Bürgel N, Fromm M, Zeitz M, Fuss I, Strober W, Schulzke JD. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* 2005; **129**: 550-564

3 **Monteleone G**, Biancone L, Marasco R, Morrone G, Marasco O, Luzza F, Pallone F. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* 1997; **112**: 1169-1178

4 **Parronchi P**, Romagnani P, Annunziato F, Sampognaro S, Becchio A, Giannarini L, Maggi E, Pupilli C, Tonelli F, Romagnani S. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol* 1997; **150**: 823-832

5 **Trinchieri G**. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003; **3**: 133-146

6 **Berrebi D**, Besnard M, Fromont-Hankard G, Paris R, Mougenot JF, De Lagausie P, Emilie D, Cezard JP, Navarro J, Peuchmaur M. Interleukin-12 expression is focally enhanced in the gastric mucosa of pediatric patients with Crohn's disease. *Am J Pathol* 1998; **152**: 667-672

7 **Presky DH**, Yang H, Minetti LJ, Chua AO, Nabavi N, Wu CY, Gately MK, Gubler U. A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc Natl Acad Sci USA* 1996; **93**: 14002-14007

8 **Szabo SJ**, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 1997; **185**: 817-824

9 **Ozenci V,** Pashenkov M, Kouwenhoven M, Rinaldi L, Söderström M, Link H. IL-12/IL-12R system in multiple sclerosis. *J Neuroimmunol* 2001; **114**: 242-252

10 **De Benedetti F**, Pignatti P, Biffi M, Bono E, Wahid S, Ingegnoli F, Chang SY, Alexander H, Massa M, Pistorio A, Martini A, Pitzalis C, Sinigaglia F, Rogge L. Increased expression of alpha(1,3)-fucosyltransferase-VII and P-selectin binding of synovial fluid T cells in juvenile idiopathic arthritis. *J Rheumatol* 2003; **30**: 1611-1615

11 **Parrello T**, Monteleone G, Cucchiara S, Monteleone I, Sebkova L, Doldo P, Luzza F, Pallone F. Up-regulation of the IL-12 receptor beta 2 chain in Crohn's disease. *J Immunol* 2000; **165**: 7234-7239

12 **Simpson SJ,** Shah S, Comiskey M, de Jong YP, Wang B, Mizoguchi E, Bhan AK, Terhorst C. T cell-mediated pathology in two models of experimental colitis depends predominantly on the interleukin 12/Signal transducer and activator of transcription (Stat)-4 pathway, but is not conditional on interferon gamma expression by T cells. *J Exp Med* 1998; **187**: 1225-1234

13 **Wirtz S**, Finotto S, Kanzler S, Lohse AW, Blessing M, Lehr HA, Galle PR, Neurath MF. Cutting edge: chronic intestinal inflammation in STAT-4 transgenic mice: characterization of disease and adoptive transfer by TNF- plus IFN-gamma-producing CD4+ T cells that respond to bacterial antigens. *J Immunol* 1999; **162**: 1884-1888

14 **Monteleone G**, Parrello T, Luzza F, Pallone F. Response of human intestinal lamina propria T lymphocytes to interleukin 12: additive effects of interleukin 15 and 7. *Gut* 1998; **43**: 620-628

15 **Monteleone G**, Monteleone I, Fina D, Vavassori P, Del Vecchio Blanco G, Caruso R, Tersigni R, Alessandroni L, Biancone L, Naccari GC, MacDonald TT, Pallone F. Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease. *Gastroenterology* 2005; **128**: 687-694

16 **Monteleone G**, Trapasso F, Parrello T, Biancone L, Stella A, Iuliano R, Luzza F, Fusco A, Pallone F. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol* 1999; **163**: 143-147

17 **Pizarro TT**, Michie MH, Bentz M, Woraratanadharm J, Smith MF Jr, Foley E, Moskaluk CA, Bickston SJ, Cominelli F. IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. *J Immunol* 1999; **162**: 6829-6835

18 **Prehn JL**, Mehdizadeh S, Landers CJ, Luo X, Cha SC, Wei P, Targan SR. Potential role for TL1A, the new TNF-family member and potent costimulator of IFN-gamma, in mucosal inflammation. *Clin Immunol* 2004; **112**: 66-77

19 **Bamias G,** Mishina M, Nyce M, Ross WG, Kollias G, Rivera-Nieves J, Pizarro TT, Cominelli F. Role of TL1A and its receptor DR3 in two models of chronic murine ileitis. *Proc Natl Acad Sci USA* 2006; **103**: 8441-8446

20 **Sato T**, Nakai T, Tamura N, Okamoto S, Matsuoka K, Sakuraba A, Fukushima T, Uede T, Hibi T. Osteopontin/Eta-1 upregulated in Crohn's disease regulates the Th1 immune response. *Gut* 2005; **54**: 1254-1262

21 **Peng SL.** The T-box transcription factor T-bet in immunity and autoimmunity. *Cell Mol Immunol* 2006; **3**: 87-95

22 **Neurath MF**, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, Mizoguchi A, Mizoguchi E, Mudter J, Galle PR, Bhan A, Autschbach F, Sullivan BM, Szabo SJ, Glimcher LH, Blumberg RS. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* 2002; **195**: 1129-1143

23 **Duchmann R**, Neurath MF, Meyer zum Büschenfelde KH. Responses to self and non-self intestinal microflora in health and inflammatory bowel disease. *Res Immunol* 1997; **148**: 589-594

24 **Duchmann R**, Schmitt E, Knolle P, Meyer zum Büschenfelde KH, Neurath M. Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. *Eur J Immunol* 1996; **26**: 934-938

25 **Lodes MJ,** Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM. Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 2004; **113**: 1296-1306

26 **Agrawal S**, Agrawal A, Doughty B, Gerwitz A, Blenis J, Van Dyke T, Pulendran B. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol* 2003; **171**: 4984-4989

27 **Watanabe T**, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* 2004; **5**: 800-808

28 **Strober W**, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* 2006; **6**: 9-20

29 **Ogura Y**, Lala S, Xin W, Smith E, Dowds TA, Chen FF, Zimmermann E, Tretiakova M, Cho JH, Hart J, Greenson JK, Keshav S, Nuñez G. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 2003; **52**: 1591-1597

30 **Cobrin GM,** Abreu MT. Defects in mucosal immunity leading to Crohn's disease. *Immunol Rev* 2005; **206**: 277-295

31 **Monteleone G,** MacDonald TT, Wathen NC, Pallone F, Pender SL. Enhancing Lamina propria Th1 cell responses with interleukin 12 produces severe tissue injury. *Gastroenterology* 1999; **117**: 1069-1077

32 **Saarialho-Kere UK**, Vaalamo M, Puolakkainen P, Airola K, Parks WC, Karjalainen-Lindsberg ML. Enhanced expression of matrilysin, collagenase, and stromelysin-1 in gastrointestinal ulcers. *Am J Pathol* 1996; **148**: 519-526

33 **Neurath MF,** Fuss I, Kelsall BL, Stüber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med* 1995; **182**: 1281-1290

34 **Fuss IJ,** Marth T, Neurath MF, Pearlstein GR, Jain A, Strober W. Anti-interleukin 12 treatment regulates apoptosis of Th1 T cells in experimental colitis in mice. *Gastroenterology* 1999; **117**: 1078-1088

35 **Mannon PJ**, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, Dolin B, Goodman N, Groden C, Hornung RL, Quezado M, Yang Z, Neurath MF, Salfeld J, Veldman GM, Schwertschlag U, Strober W. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 2004; **351**: 2069-2079

36 **Fuss IJ**, Becker C, Yang Z, Groden C, Hornung RL, Heller F, Neurath MF, Strober W, Mannon PJ. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis* 2006; **12**: 9-15

37 **Hommes DW**, Mikhajlova TL, Stoinov S, Stimac D, Vucelic B, Lonovics J, Zákuciová M, D'Haens G, Van Assche G, Ba S, Lee S, Pearce T. Fontolizumab, a humanised anti-interferon gamma antibody, demonstrates safety and clinical activity in patients with moderate to severe Crohn's disease. *Gut* 2006; **55**: 1131-1137

38 **Hunter CA**. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 2005; **5**: 521-531

39 **Schmidt C**, Giese T, Ludwig B, Mueller-Molaian I, Marth T, Zeuzem S, Meuer SC, Stallmach A. Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm Bowel Dis* 2005; **11**: 16-23

40 **Yen D**, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 2006; **116**: 1310-1316

41 **Mathur AN**, Chang HC, Zisoulis DG, Kapur R, Belladonna ML, Kansas GS, Kaplan MH. T-bet is a critical determinant in the instability of the IL-17-secreting T-helper phenotype. *Blood* 2006; **108**: 1596-1601

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REVIEW

Upper gastrointestinal function and glycemic control in diabetes mellitus

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Abstract

Recent evidence has highlighted the impact of glycemic control on the incidence and progression of diabetic micro- and macrovascular complications, and on cardiovascular risk in the non-diabetic population. Postprandial blood glucose concentrations make a major contribution to overall glycemic control, and are determined in part by upper gastrointestinal function. Conversely, poor glycemic control has an acute, reversible effect on gastrointestinal motility. Insights into the mechanisms by which the gut contributes to glycemia have given rise to a number of novel dietary and pharmacological strategies designed to lower postprandial blood glucose concentrations.

Key words: Blood glucose; Diabetes mellitus; Gastric Emptying; Gastrointestinal motility; Hyperglycemia

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INTRODUCTION

Diabetes and its long-term complications, which include cardiovascular, renal, neurologic, and ophthalmic disease, represent a major cause of morbidity and mortality throughout the world[1]. The prevalence of both type 1 (insulin-dependant) and type 2 (non insulin-dependant) diabetes is increasing, the latter as a consequence of obesity. In the US, 29 million people and 14% of adults have diabetes or impaired fasting glucose, of whom about a third are undiagnosed[2]. Similar figures are evident throughout the developed world[3].

Hyperglycemia is central to the pathogenesis of diabetic micro- and macrovascular complications[4]. There is increasing evidence that postprandial hyperglycemia is the major determinant of "average" glycemic control, and represents an independent risk factor for macrovascular disease, even in people without diabetes[5]. While the relative importance of individual determinants of the blood glucose response to a meal remain to be clarified precisely, it is clear that upper gastrointestinal motility has a major impact that has generally been overlooked. Moreover, postprandial glycemic control in turn has a profound effect on the motor function of the upper gut. Hence, blood glucose concentration is determined by, as well as a determinant of, gastric and small intestinal motility[6].

The aims of this review are to examine (1) evidence relating to the importance of postprandial, as opposed to fasting, glycemia in the development and progression of diabetic complications, (2) the contribution of upper gastrointestinal function to postprandial blood glucose concentrations, (3) the impact of variations in glycemia on gastric and small intestinal motility, and (4) the therapeutic strategies suggested by these insights.

IMPACT OF POSTPRANDIAL GLYCEMIA ON COMPLICATIONS OF DIABETES

The DCCT/EDIC and UKPDS trials established that the onset and progression of microvascular, and probably macrovascular complications of diabetes, are related to "average" glycemic control, as assessed by glycated hemoglobin[4,7,8]. This provides a rationale for the widespread use of intensive therapy directed at the normalisation of glycemia. In the recently reported DCCT/EDIC study, a period of intensive, as opposed to conventional, therapy for a period of 6.5 years between 1983 and 1993 was shown to be associated with a reduction in the risk of a subsequent cardiovascular event by 42%[4]. Glycated hemoglobin is potentially influenced by both fasting and postprandial blood glucose concentrations. However, given that the stomach empties ingested nutrients at a closely regulated overall rate of 2-3 kcal per minute[9,10] and humans ingest around 2500 kcal daily, it is clear that most individuals spend the majority of each day in either the postprandial or post-absorptive phase with a limited duration of true "fasting" for perhaps three or four hours before breakfast[11]. Hence, the traditional focus on the control of "fasting" blood glucose in diabetes management appears inappropriate.

It is well established that postprandial hyperglycemia precedes elevation of fasting blood glucose in the

evolution of diabetes[12]. Furthermore, it appears to be the better predictor of coronary artery[13] and cerebrovascular[14] complications, and predicts all-cause and cardiovascular mortality, even in the general population without known diabetes[15]. Blood glucose values at two hours during an oral glucose tolerance test are a better predictor of mortality than fasting blood glucose[16], and reduction in cardiovascular risk in patients with type 2 diabetes is associated with control of postprandial, as opposed to fasting, glycemia[17]. For example, patients with impaired glucose tolerance who were treated with the α-glucosidase inhibitor acarbose, to reduce postprandial glycemia, experienced a reduction in cardiovascular risk of about a third compared to placebo during a mean of three years follow-up[18]. Postprandial blood glucose concentrations correlate well with glycated hemoglobin in the setting of mild to moderate hyperglycemia[19], with fasting blood glucose only assuming greater importance at higher HbA1c values[20]. There is also evidence that therapy directed towards lowering postprandial blood glucose concentrations may have a greater impact on HbA1c than attention to fasting blood glucose[21].

Hyperglycemia potentially has diverse effects on blood vessels. In the short term, hyperglycemia is associated with activation of protein kinase C, which affects endothelial permeability, cell adhesion, and proliferation in the vessel wall. Over the longer term, non-enzymatic glycosylation of proteins results in atherosclerosis[22]. Elevated postprandial blood glucose concentrations are associated with an increase in plasma biochemical markers of oxidative stress[23,24]. However, to what degree hyperglycemia *per se* accounts for this effect, as opposed to concurrent elevations of non-esterified fatty acids and triglycerides, remains to be elucidated[25].

CONTRIBUTION OF UPPER GASTROINTESTINAL FUNCTION TO POSTPRANDIAL GLYCEMIA

Postprandial blood glucose levels are potentially affected by a number of factors, including pre-prandial blood glucose concentration, food properties such as viscosity, fibre content, and quantity and type of carbohydrate, gastric emptying, small intestinal delivery and absorption of nutrients, insulin secretion, hepatic glucose metabolism, and peripheral insulin sensitivity[26]. The relative importance of these factors is likely to vary with time after a meal, and between healthy subjects and patients with type 1 or type 2 diabetes. While it is logical that the gastrointestinal tract, which controls the rate at which ingested carbohydrate is absorbed and releases peptides that stimulate insulin secretion, should have a major impact on postprandial glycemia, its role has frequently been overlooked and generally underestimated in the past. The rate of gastric emptying is now established as a major contributor to variations in glycemia, while the influence of small intestinal motor function represents a current research focus.

Gastric emptying

Gastric emptying accounts for at least 35% of the variance

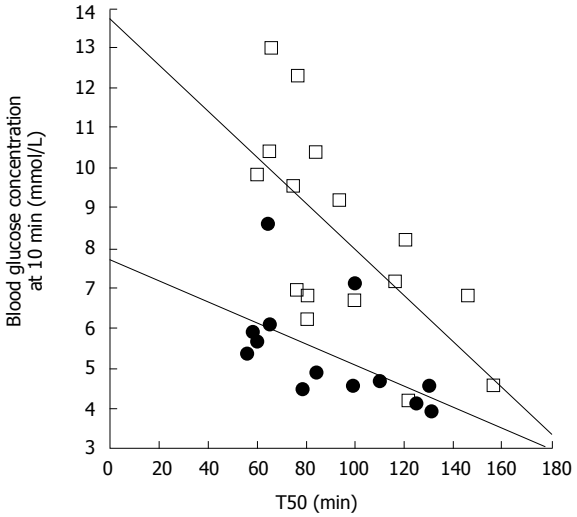

Figure 1 Relationship between the blood glucose concentration 10 min after consuming 75 g glucose in 300 mL water, and the gastric half-emptying time (T50) in patients with type 2 diabetes (open squares, r = -0.67, P < 0.005) and healthy subjects (filled circles, r = -0.56, P < 0.05). Adapted from Jones *et al* 1996[28].

in the initial rise as well as the peak blood glucose levels after an oral glucose load in both healthy individuals[27] and patients with type 2 diabetes[28] (Figure 1). Pharmacological slowing of gastric emptying by morphine reduces the postprandial glycemic response to a mixed meal in type 2 patients, whilst acceleration of gastric emptying by erythromycin (a motilin agonist) increases postprandial blood glucose concentrations (Figure 2). Here, differences in peak blood glucose values are more marked than those in the area under the blood glucose curve[29]. In type 1 patients the glycemic response to a meal, and therefore the requirement for exogenous insulin, is also critically dependent on the rate of gastric emptying. Here, when emptying is slower, the insulin requirement to achieve euglycemia is less[30].

In health, gastric emptying is modulated by feedback arising from the interaction of nutrients with the small intestine, so that the overall rate of gastric emptying is closely regulated at about 2-3 kcal per minute[9]. Infusion of a caloric load directly into the small intestine slows gastric emptying by a mechanism that includes relaxation of the gastric fundus, suppression of antral motility, and stimulation of phasic and tonic pressures in the pylorus[31,32]; the latter acts as a brake to gastric outflow. The length of small intestine exposed to nutrient appears to be the primary determinant of the magnitude of the feedback response[33,34]. Both transection of duodenal intramural nerves[35], and suppression of the release of small intestinal peptides by the somatostatin agonist, octreotide[36], accelerate the emptying of nutrient liquids, indicating the involvement of both neural and humoral mechanisms in mediating feedback from the small intestine. Glucagon like peptide-1 (GLP-1), which suppresses antral and duodenal motility and stimulates pyloric contractions[37-39], probably represents one such humoral mediator, and the slowing of gastric emptying by this peptide appears likely to be the major mechanism by which its administration improves postprandial glycemia in patients with type 2 diabetes[40]. While the contribution of endogenous GLP-1 in regulating gastric emptying

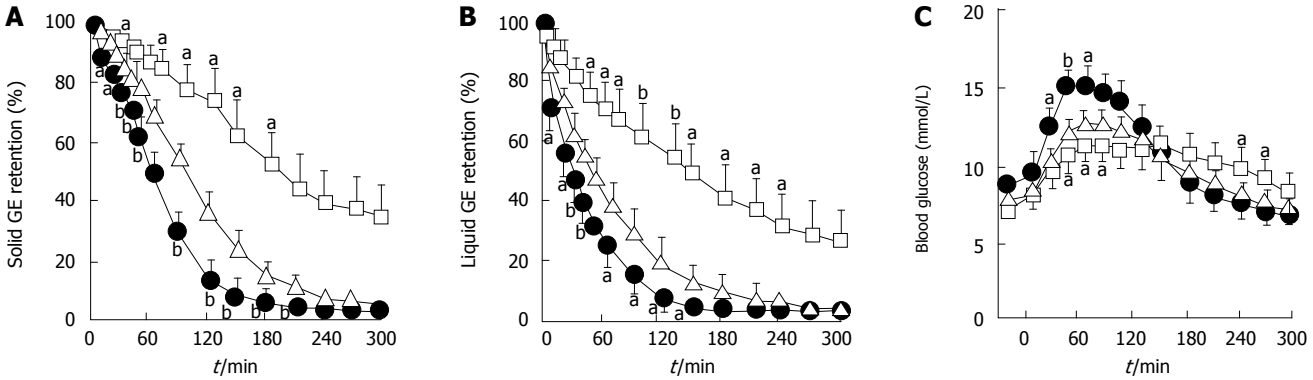

Figure 2 Effects of erythromycin (200 mg iv, filled circles) and morphine (8 mg iv, open squares) compared to placebo (open triangles) on (**A**) solid and (**B**) liquid gastric emptying and (**C**) blood glucose concentration in 9 patients with type 2 diabetes. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* placebo. Adapted from Gonlachanvit *et al* 2003[29].

remains to be clarified (i.e. the "physiological" as opposed to "pharmacological" action), a recent study using the GLP-1 antagonist, exendin (9-39) amide, confirmed that endogenous GLP-1 mediates the suppression of antral motility and stimulation of pyloric pressures induced by the presence of glucose in the small intestine[38].

It has long been recognised that oral or enteral administration of glucose results in a much greater insulin response than an equivalent intravenous glucose load[41-44], a phenomenon referred to as the "incretin" effect. The putative incretin peptides, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), are released from the small intestine in response to nutrients[45], apparently in a load-dependent fashion[46]. Therefore, the rate of delivery of carbohydrate from the stomach into the small intestine is likely to be critical in determining not only the rate of glucose absorption, but also the incretin response. Although GIP is the more potent of the two hormones in healthy individuals[47], the insulinotropic effect of GIP appears to be markedly diminished in patients with type 2 diabetes, while the insulin response to GLP-1 is retained[45]. This forms a rationale for the therapeutic use of GLP-1 and its analogs in the management of type 2 diabetes (to be discussed). There is limited evidence that type 2 diabetes is associated with an impaired GLP-1 response to oral glucose[48], but to what degree delayed gastric emptying, which occurs frequently in type 2 patients[49], accounts for this decrease in GLP-1 has not been evaluated.

In considering the potential impact of gastric emptying on postprandial glycemia, the initial rate of glucose entry into the small intestine ("early phase" of gastric emptying) may be particularly important[50]. Type 2 diabetes is characterised by a reduced "early", and frequently increased "late", postprandial insulin response. Studies in rodents have established the importance of "early" insulin release as a determinant of postprandial glucose excursions, in that a small "early" increase in portal vein/peripheral blood insulin is more effective than a larger, "later" increase at reducing blood glucose levels[51]. A recent study involving both healthy subjects and type 2 patients, established that modest physiological variations in the initial rate of small intestinal glucose entry have major effects on the subsequent glycemic, insulin and incretin responses (Figure 3)[52]. Nevertheless, while initially rapid, and subsequently slower, duodenal glucose delivery can boost incretin and insulin responses when compared to constant delivery of an identical glucose load, the overall glycemic excursion is, if anything, greater[52], and certainly not improved[53]. This evidence adds to the rationale for the use of dietary and pharmacological strategies designed to reduce postprandial glycemic excursions by slowing gastric emptying, rather than initially accelerating it. However, the "dose-response" relationship between duodenal glucose delivery and glycemia remains to be clearly determined.

Small intestinal glucose absorption

The gut lumen is the site of absorption of glucose from the external environment into the body, as well as being the source of the incretin peptides that drive much of the postprandial insulin response. Thus, it is logical that variations in small intestinal function should be a major determinant of postprandial glycemia. Nevertheless, there is little information about the impact of small intestinal motility and absorptive function on glycemia, at least in part because of the technical demands in studying this region of gastrointestinal tract[6,54].

The large surface area of the small intestine is well suited to absorption of water and solutes. Perfusion studies in healthy humans have established that the proximal jejunum has a maximal absorptive capacity for glucose of approximately 0.5 g per minute per 30 cm[55-57]. Small intestinal mucosal hypertrophy occurs in animal models of diabetes, with concomitant increases in glucose absorption, but this is rapidly reversed by insulin treatment[58]. However, acute hyperglycemia does result in transient increases in intestinal glucose absorption in rodents[59-61]. The few studies performed in humans with type 1[62] "insulin-requiring"[63], or type 2[64] diabetes have not demonstrated increased small intestinal glucose absorption, other than one report of increased absorption at high luminal glucose concentrations[65]. Attention was paid to maintaining euglycemia in at least one of these studies[63]. However, there is a recent report of increased expression of monosaccharide transporters in humans with type 2 diabetes[66], the clinical significance of which remains to be clarified. One human study failed to demonstrate an effect of marked hyperglycemia (14 mmol/L) on jejunal glucose absorption in healthy subjects[63], although a relationship has been observed between more physiological postprandial blood glucose concentrations (less than 10 mmol/L) and the absorption of the glucose analog, 3-O-methylglucose, in healthy

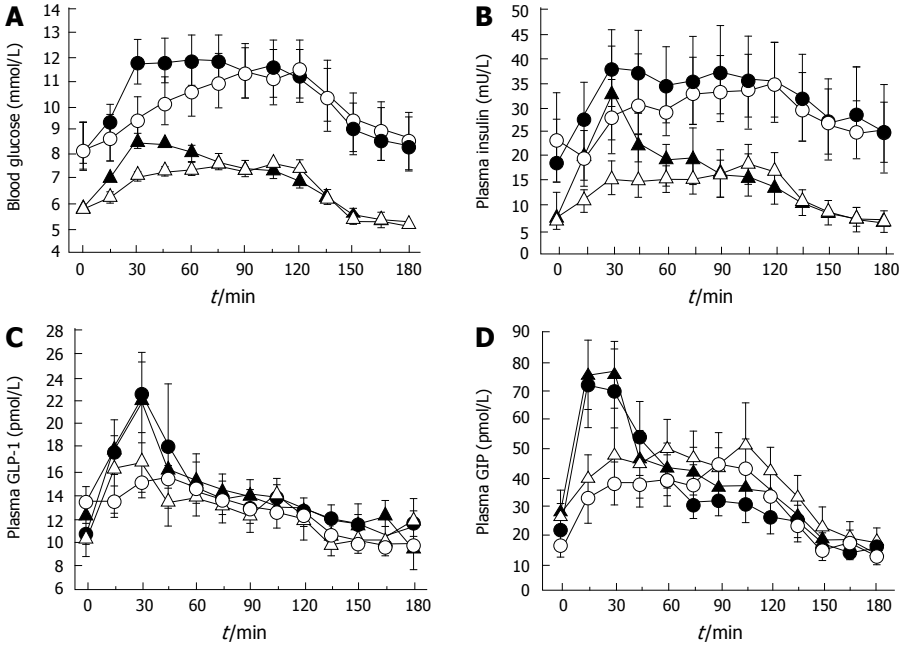

Figure 3 Effect of initially more rapid intraduodenal glucose infusion (3 kcal/min between t = 0 and 15 min and 0.71 kcal/min between t = 15 and 120 min) (closed symbols) compared to constant infusion (1 kcal/min between t = 0 and 120 min) (open symbols) in healthy subjects (triangles) and patients with type 2 diabetes (circles) on (**A**) blood glucose, (**B**) plasma insulin, (**C**) plasma GLP-1, and (**D**) plasma GIP. Each pair of curves differs between 0 and 30 min for variable *vs* constant intraduodenal infusion ($P < 0.05$). Adapted from O'Donovan *et al* 2004[52].

subjects and patients with type 1 diabetes[67]. Hence, the effect of transient hyperglycemia on small intestinal glucose absorption remains uncertain.

Given that an upper limit exists for absorption of glucose across the small intestinal mucosa, it is logical that patterns of intestinal motility which serve to spread luminal glucose over a large surface area could promote glucose absorption. Furthermore, certain motor patterns could facilitate mixing of complex carbohydrates with digestive enzymes, and their exposure to brush border disaccharidases. Thus, when glucose is infused directly into the duodenum, its rate of absorption increases with the number of duodenal pressure waves and propagated pressure wave sequences[54,67]. Further insights into the effects of luminal flow on glucose absorption are likely to require additional techniques, such as intraluminal impedance measurement, in which inferences can be made regarding movement of fluid boluses by measuring changes in electrical impedance between sequential pairs of intraluminal electrodes. A preliminary study in healthy humans using this technique indicates that pharmacological suppression of intraduodenal flow with an anticholinergic agent is associated with delayed absorption of luminal glucose[68]. These observations are likely to be of relevance to patients with type 1 diabetes mellitus, who demonstrate an increased frequency of small intestinal pressure waves in the postprandial state[67].

The region of small intestine that is exposed to carbohydrate is also likely to be a determinant of the glycemic response. GLP-1 is released from intestinal L cells, whose concentration is greatest is the distal jejunum, with fewer L cells located in the proximal jejunum, ileum, and colon[69]. In humans, it is unclear whether nutrients must interact directly with L cells to stimulate GLP-1 release. A neural or endocrine loop between the duodenum and the more distal small bowel has been postulated[70], but remains unproven. The lack of a GLP-1 response when glucose is infused into the duodenum below a certain dose (1.4 kcal/min)[71] would be consistent with the concept of complete absorption of the glucose load high in the small intestine, precluding significant interaction with L cells, although other investigators have reported a GLP-1 response even with 1 kcal/min intraduodenal glucose infusion[52]. Nevertheless, GLP-1 responses to meals are enhanced following intestinal bypass procedures that promote access of nutrients to more distal small intestine[72-75], while inhibition of sucrose digestion in the proximal small intestine with acarbose increases the GLP-1 response[76], presumably by facilitating more distal interaction of the intestine with glucose. It follows that dietary modifications that favor exposure of more distal small intestinal segments to glucose could reduce glycemic excursions by stimulating GLP-1 release. Furthermore, a major action of both GLP-1 and peptide YY, which is also released from L cells, is to retard gastric emptying, thus slowing any further entry of carbohydrate to the small intestine[77].

IMPACT OF VARIATIONS IN GLYCEMIA ON UPPER GUT MOTILITY

Acute changes in the blood glucose concentration are now recognised to have a major, reversible impact on the motor function of every region of the gastrointestinal tract. This may in part account for the poor correlation of upper gastrointestinal dysfunction in diabetes with evidence of irreversible autonomic neuropathy, to which it has traditionally been attributed[78]. When compared to euglycemia (4-6 mmol/L), gut motility is modulated through the range of blood glucose concentrations from marked hyperglycemia (greater than 12 mmol/L), through "physiological" blood glucose elevation (8-10 mmol/L), to insulin-induced hypoglycemia (less than 2.5 mmol/L), and effects are observed rapidly (within minutes), although the thresholds of response may differ between gut regions[6]. The mechanisms mediating the effects of acute changes in the blood glucose concentration are poorly defined, and the potential impact of chronic, as opposed to acute, variations in glycemia on gastrointestinal motility has hitherto received little attention. Nevertheless, it is clear

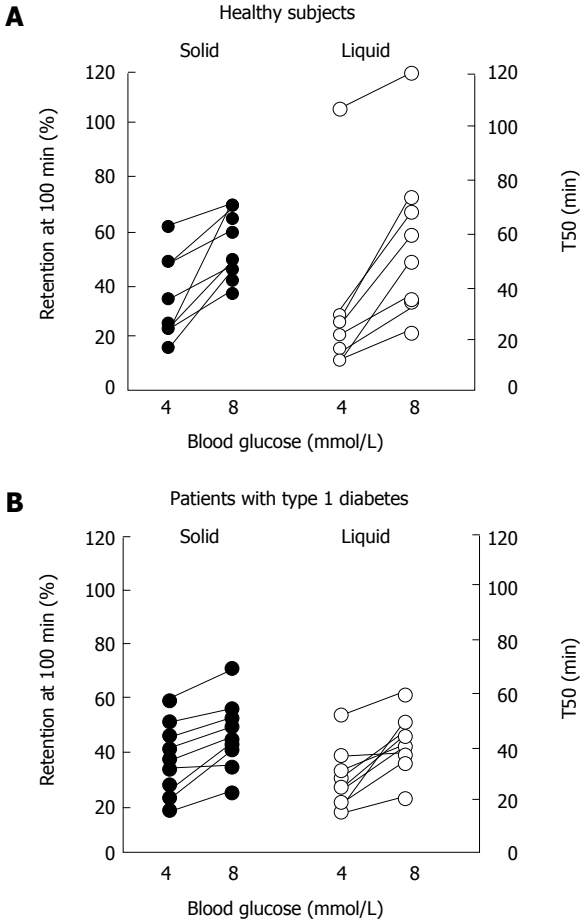

Figure 4 Solid and liquid gastric emptying in (**A**) healthy subjects and (**B**) patients with type 1 diabetes mellitus during euglycemia (blood glucose 4 mmol/L) and "physiological" hyperglycemia (blood glucose 8 mmol/L). Adapted from Schvarcz *et al* 1997[82].

that gut motor function and postprandial glycemia are highly interdependent variables.

Gastric motility

Marked hyperglycemia (16-20 mmol/L) slows both solid and nutrient liquid emptying in patients with type 1 diabetes when compared to euglycemia (5-8 mmol/L)[79]; in type 2 patients, cross-sectional data also indicate an inverse relationship between the blood glucose concentration and the rate of gastric emptying[49]. Conversely, gastric emptying is accelerated by acute hypoglycemia induced by insulin (2.6 mmol/L) in healthy subjects[80] and type 1 patients, even when emptying is slower than normal during euglycemia[81]. In type 1 diabetes, as well as healthy volunteers, elevation of blood glucose to "physiological" postprandial levels (8 mmol/L) also slows gastric emptying when compared to euglycemia (4 mmol/L)[82] (Figure 4). The magnitude of the effects of glycemia on the rate of gastric emptying is substantial, and has implications for absorption of orally administered medications, including oral hypoglycemic agents[83], as well as impacting on carbohydrate absorption.

The rate of gastric emptying is determined by the coordinated activity of various regions of the stomach and proximal small intestine[84]. The proximal stomach acts as a reservoir for solids and generates tonic pressure to facilitate liquid emptying. The distal stomach grinds and sieves solids and pumps chyme across the pylorus, predominantly in a pulsatile manner, while phasic and tonic pyloric pressures, and duodenal contractile activity act as a break to gastric outflow. The timing of antral contractions is controlled by an electrical slow wave, with a frequency of about 3 per minute, generated by the interstitial cells of Cajal[85]. During fasting, cyclical activity of gastric motility is observed, with a periodicity of about 90 min, characterised by irregular contractions of increasing frequency (phase Ⅱ), and a brief (5-10 min) period of regular contractions at the rate of 3 per minute (phase Ⅲ) during which indigestible solids empty from the stomach, followed by motor quiescence (phase Ⅰ). Acute hyperglycemia is associated with diminished proximal gastric tone[86-88], suppression of both the frequency and propagation of antral pressure waves[89-92], and stimulation of pyloric contractions[93] -a motor pattern associated with slowing of gastric emptying. The frequency of the gastric slow wave is also disturbed[94]. The suppression of antral motility is observed at blood glucose concentrations as low as 8 mmol/L[89,91]; the threshold appears higher in the proximal stomach[95]. Hyperglycemia also attenuates the prokinetic effects of erythromycin in both healthy subjects and type 1 patients[96,97], at least in part by inhibiting the stimulation of antral waves and coordinated antroduodenal pressure sequences[98]. This effect is of considerable importance, since the action of other prokinetic drugs is also likely to be impaired during hyperglycemia, although this issue has not been specifically examined.

Small intestinal motility

As in the stomach, fasting small intestinal motility is cyclical, and characterised by phases Ⅰ to Ⅲ, the latter with a frequency of 9 to 12 per minute. This "migrating motor complex" (MMC) propagates aborally along the small intestine, and serves to "sweep" the lumen of indigestible debris. After a meal, the MMC is interrupted by irregular contractions propagated over short distances, which facilitate digestion and absorption of nutrients.

In healthy subjects during hyperglycemia (10 mmol/L), the duodenum becomes less compliant (more "stiff") to balloon distension, while distension stimulates a greater number of phasic pressure waves, when compared to euglycemia[99]. Both phenomena could contribute to a duodenal "brake" to gastric emptying. More marked hyperglycemia (12-15 mmol/L) reduces the cycle length of the MMC[100], the frequency of duodenal and jejunal pressure waves, and the duration of the postprandial period (early return of phase Ⅲ activity), and slows small intestinal transit[101]. These alterations in function could have implications for absorption of nutrients and medications, alteration in bowel habit, and the occurrence of small intestinal bacterial overgrowth in diabetes[102]. However, other than suppression of proximal duodenal wave frequency[103], there is limited information about the effects of hyperglycemia on small intestinal motor function in patients, as opposed to healthy volunteers.

Mechanisms mediating the effects of hyperglycemia on gastric and small intestinal motility

Most information about the etiology of gastrointestinal dysfunction in diabetes relates to the effects of

longstanding diabetes rather than acute, reversible changes that could relate to transient hyperglycemia[104]. Rodent models of diabetes have demonstrated marked apoptosis of enteric neurons[105], affecting nitrergic (inhibitory) neurons in particular[106], and loss of interstitial cells of Cajal[107]. The latter are also deficient in humans with diabetes and severe gut symptoms[108]. Hyperglycemia appears to be responsible for apoptosis of enteric neurons[109], but the latter would seem unlikely to mediate changes that are evident within minutes, rather than days or weeks. Enteric neurons sensitive to changes in glucose have been identified[110], although their responsiveness to systemic, as opposed to luminal glucose remains unclear. Vagal nerve function is reversibly inhibited by acute hyperglycemia[111,112], and this may account for some of the observed phenomena. Hyperinsulinemia is unlikely to explain the observed effects, particularly as they are seen in type 1 (insulin deficient) as well as type 2 and healthy subjects. Studies are indicated to determine whether reversible changes in nitrergic or serotonergic neurotransmission occur with variations in glycemia.

THERAPEUTIC STRATEGIES DIRECTED AT MINIMISING POSTPRANDIAL GLYCEMIA

The major impact of gastrointestinal function on the glycemic response to meals, as outlined above, suggests a number of logical, and in many cases complimentary, strategies to lower postprandial blood glucose concentrations (Table 1). These include (1) minimising the carbohydrate content, or substituting low- for high-glycemic index foods in meals, (2) slowing gastric emptying, even in individuals with modest delays in emptying provided they remain free of symptoms, (3) inhibiting the absorption of carbohydrate from the small intestine, or delaying its absorption to more distal small intestinal segments, and (4) augmenting the incretin response. Many approaches fulfill a number of these aims concurrently. Most studies relating elevated postprandial glycemia with cardiovascular risk have evaluated blood glucose 2 h after a meal[113], suggesting that lowering peak blood glucose may be an appropriate target. Nevertheless, the glycated hemoglobin relates closely to the integrated mean blood glucose (i.e. area under the curve) over 24 h, albeit with a curvilinear rather then linear relationship[114], so reducing the total area under the blood glucose excursion over several hours after a meal may also be an important goal. It should be noted that strategies for individuals with impaired glucose tolerance or type 2 diabetes managed without exogenous insulin, particularly those involving slowing of gastric emptying, may not be applicable to type 1 and insulin-requiring type 2 patients, in whom the goal should be to coordinate the absorption of carbohydrate with the action of exogenous insulin, which in some cases may involve accelerating gastric emptying, if it is already delayed[115].

Table 1 Therapeutic strategies directed at minimizing postprandial glycemia

Strategy	Examples
Alter carbohydrate in diet	Low-carbohydrate diets
	Low glycemic index carbohydrates
Slow gastric emptying	High fiber diets
	Fat "preloads"
	Low glycemic index carbohydrates
	Acarbose
	GLP-1 analogs and DPP-IV inhibitors
	Pramlintide
Inhibit small intestinal carbohydrate absorption	Acarbose
	High fiber diets
	Low glycemic index carbohydrates
Augment the incretin response	GLP-1 analogs and DPP-IV inhibitors
	Acarbose
	Fat "preloads"
	? Low glycemic index carbohydrates

Carbohydrate content and glycemic index

Low-carbohydrate diets were the mainstay of treatment for diabetes in the pre-insulin era[116]. The outcome of the Nurses' Health Study is indicative of a relationship between both cardiovascular risk and the incidence of diabetes with dietary glycemic load[117]. Short-term studies indicate the potential for low-carbohydrate diets to improve 24 h glycemia and glycated hemoglobin in patients with type 2 diabetes[118], including those failing conventional treatment with diet and a sulfonylurea[119]. In medium- to long-term studies, substituting protein for carbohydrate improved glycemia in overweight hyperinsulinemic subjects[120], while a low-carbohydrate diet improved fasting glucose over 6 mo in type 2 patients, with glycemic benefits maintained at 1 year, when compared to a low-fat diet[121,122]. The magnitude of the decrease in glycated hemoglobin was small (mean 0.6% in the latter study), but likely to be clinically significant. In addition to the reduction in carbohydrate load, protein itself might improve glycemia by stimulating insulin release[123], although this phenomenon is less apparent in medium- versus short-term studies[124].

Rather than trading carbohydrates for alternative macronutrients, another approach is to substitute low- for high-glycemic index carbohydrates. The glycemic index (GI) compares the blood glucose response of a test food with that of a standard carbohydrate, either glucose or white bread[125]. Foods may be low GI by virtue of a relative delay in gastric emptying and/or small intestinal glucose absorption[126,127]. For example, spaghetti (low GI) empties from the stomach much slower than potato (high GI) from about 60 min after the meal, although their glycemic profiles diverge earlier[128], indicating that slowing of small intestinal glucose absorption is important. Both the physical properties of the carbohydrate (such as enclosed kernels) and its chemical composition (such as a high amylose:amylopectin ratio) influence small intestinal carbohydrate digestion and absorption[127,129]. Glycemic index tends to vary inversely with the content of dietary fiber in meals[130]; dietary fiber *per se* potentially slows gastric emptying[131] and small intestinal carbohydrate absorption[132], the latter by a mechanism that includes modification of small intestinal motility from a stationary (favoring mixing), to a propulsive pattern. The beneficial effect on the glycemic response of adding guar gum to an oral glucose load appears to be achieved mainly by slowing gastric emptying[133,134]. Nevertheless, guar also slows

small intestinal glucose absorption, probably by inhibiting diffusion of glucose out of the luminal contents[135]; this is reflected in the observation that both GLP-1 and insulin responses are less than with guar-free glucose[136].

Low GI foods may also stimulate insulin release through the incretin effect, or other mechanisms[137]. Furthermore, they may enhance satiety and reduce energy intake at a subsequent meal[138,139]. Additional information about the potential for these beneficial effects from different classes of low GI foods is needed. Fructose has been advocated as a low GI substitute for glucose in the diabetic diet, since it results in a much lower glycemic excursion than an equivalent glucose load[140], while retaining the capacity to stimulate insulin secretion[141]. In addition, some investigators have found that fructose ingestion suppresses food intake more than glucose[140,142], although this issue is controversial[141], and probably depends on the load and timing of fructose ingestion in relation to the subsequent meal.

Most medium- to long-term studies of low GI diets indicate a benefit for glycemic control[143]; typically the effect is modest[144], but is still likely to be clinically meaningful, and in many cases is of similar magnitude to the improvement in glycemic control achieved by pharmacological agents[145].

Slowing gastric emptying

Given the relationship between the degree of postprandial glycemia and the rate of gastric emptying in both healthy subjects[27] and type 2 patients[28], it is logical that dietary and/or pharmacological interventions which slow gastric emptying should be effective in lowering postprandial glycemia in type 2 diabetes. In addition to the effects of dietary fiber in retarding gastric emptying, slowing of emptying by either an oral proteinase inhibitor[146], adding a solid non-carbohydrate meal to an oral glucose load[147], or combining fat (the most potent macronutrient for slowing gastric emptying[148]) with carbohydrate[149], all reduce postprandial blood glucose and insulin responses. The underlying concept is that the presence of nutrients in the small intestine both delays gastric emptying and stimulates GLP-1 and GIP. Hence, consumption of oil as a "preload" before a mashed potato meal markedly delays the subsequent rise in blood glucose, and stimulates GLP-1 release in patients with type 2 diabetes[150]. This approach requires further refinement to determine the optimum load, timing, and macronutrient content of the "preload", but has the potential advantage of simplicity when compared to pharmacological strategies, which also appear to act predominantly by slowing gastric emptying. For example, the improvement in postprandial glycemia associated with GLP-1 and its analogs is related to slowing of gastric emptying rather than enhancement of insulin secretion[40]; the latter is in fact reduced due to a decrease in the rate of entry of carbohydrate into the small intestine. The amylin analog, pramlintide, also slows gastric emptying[151], and its use is associated with an improvement in overall glycemic control, as assessed by glycated hemoglobin, with medium-term use in type 1 and type 2 patients[152-155]. Pramlintide has the additional advantage of promoting weight loss, probably by suppressing appetite[156].

Inhibiting absorption of glucose

The α-glucosidase inhibitors, including acarbose, delay absorption of carbohydrates in the proximal small intestine[157]. The resultant exposure of more distal intestinal segments to glucose results in enhanced and prolonged GLP-1 secretion in healthy subjects[76,158], with subsequent slowing of gastric emptying[159]. The magnitude of these affects is likely to depend on meal content (ie. disaccharide load). Moreover, acarbose fails to stimulate GLP-1 release or delay gastric emptying in patients with type 2 diabetes, although it is still beneficial for reducing postprandial glycemia in this group[160], presumably by impairing carbohydrate absorption. Inhibition of glucose entry into enterocytes might represent an additional mode of action of acarbose[161]. Again, it is clear that the mechanisms by which postprandial glycemia is improved frequently overlap. For example, slowed absorption of glucose, as discussed, is also a feature of low GI and high fiber diets.

Augmenting the incretin response

The effect on GLP-1 concentrations of the dietary strategies already discussed, and the observed potentiation of GLP-1 secretion and associated improvement in glycemic control after bariatric surgery[162], point to the value of augmenting the incretin response in optimising postprandial glycemia. GLP-1 is metabolised rapidly by the enzyme dipeptidyl peptidase Ⅳ (DPP-Ⅳ), and therefore is not a suitable agent for therapeutic administration. Instead, longer lasting agonists have been developed, including both albumin-bound analogs of GLP-1, and exendin-4, a peptide derived from the saliva of the Gila monster lizard, which is structurally similar to GLP-1 and shares several biological properties, but may be a more potent insulinotropic agent than GLP-1[26]. Subcutaneous administration has been shown to reduce postprandial glycemia in type 2 patients[163]. Resistant analogs of GLP-1, along with DPP-Ⅳ inhibitors, appear to have a promising role in the therapy of diabetes[164].

CONCLUSION

Upper gastrointestinal function plays a major, though often overlooked, role in determining postprandial glycemia. Recent insights into the mechanisms by which variations in gut function alter the blood glucose concentration have suggested a number of potential therapeutic strategies that require further evaluation for their utility in achieving good glycemic control.

REFERENCES

1 **Mokdad AH**, Ford ES, Bowman BA, Nelson DE, Engelgau MM, Vinicor F, Marks JS. Diabetes trends in the U.S.: 1990-1998. *Diabetes Care* 2000; **23**: 1278-1283

2 Prevalence of diabetes and impaired fasting glucose in adults--United States, 1999-2000. *MMWR Morb Mortal Wkly Rep* 2003; **52**: 833-837

3 **Dunstan DW**, Zimmet PZ, Welborn TA, De Courten MP, Cameron AJ, Sicree RA, Dwyer T, Colagiuri S, Jolley D, Knuiman M, Atkins R, Shaw JE. The rising prevalence of diabetes and impaired glucose tolerance: the Australian Diabetes, Obesity and Lifestyle Study. *Diabetes Care* 2002; **25**: 829-834

4 **Nathan DM**, Cleary PA, Backlund JY, Genuth SM, Lachin JM, Orchard TJ, Raskin P, Zinman B. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med* 2005; **353**: 2643-2653

5 **Del Prato S**. In search of normoglycaemia in diabetes: controlling postprandial glucose. *Int J Obes Relat Metab Disord* 2002; **26** Suppl 3: S9-17

6 **Rayner CK**, Samsom M, Jones KL, Horowitz M. Relationships of upper gastrointestinal motor and sensory function with glycemic control. *Diabetes Care* 2001; **24**: 371-381

7 **The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus**. The Diabetes Control and Complications Trial Research Group. *N Engl J Med* 1993; **329**: 977-986

8 **Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)**. UK Prospective Diabetes Study (UKPDS) Group. *Lancet* 1998; **352**: 837-853

9 **Brener W**, Hendrix TR, McHugh PR. Regulation of the gastric emptying of glucose. *Gastroenterology* 1983; **85**: 76-82

10 **Hunt JN**, Smith JL, Jiang CL. Effect of meal volume and energy density on the gastric emptying of carbohydrates. *Gastroenterology* 1985; **89**: 1326-1330

11 **Monnier L**. Is postprandial glucose a neglected cardiovascular risk factor in type 2 diabetes? *Eur J Clin Invest* 2000; **30** Suppl 2: 3-11

12 **Lebovitz HE**. Postprandial hyperglycaemic state: importance and consequences. *Diabetes Res Clin Pract* 1998; **40** Suppl: S27-S28

13 **Balkau B**, Shipley M, Jarrett RJ, Pyörälä K, Pyörälä M, Forhan A, Eschwège E. High blood glucose concentration is a risk factor for mortality in middle-aged nondiabetic men. 20-year follow-up in the Whitehall Study, the Paris Prospective Study, and the Helsinki Policemen Study. *Diabetes Care* 1998; **21**: 360-367

14 **Yamasaki Y**, Kawamori R, Matsushima H, Nishizawa H, Kodama M, Kubota M, Kajimoto Y, Kamada T. Asymptomatic hyperglycaemia is associated with increased intimal plus medial thickness of the carotid artery. *Diabetologia* 1995; **38**: 585-591

15 **de Vegt F**, Dekker JM, Ruhé HG, Stehouwer CD, Nijpels G, Bouter LM, Heine RJ. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 1999; **42**: 926-931

16 **Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria**. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe. *Lancet* 1999; **354**: 617-621

17 **Hanefeld M**, Fischer S, Julius U, Schulze J, Schwanebeck U, Schmechel H, Ziegelasch HJ, Lindner J. Risk factors for myocardial infarction and death in newly detected NIDDM: the Diabetes Intervention Study, 11-year follow-up. *Diabetologia* 1996; **39**: 1577-1583

18 **Chiasson JL**, Josse RG, Gomis R, Hanefeld M, Karasik A, Laakso M. Acarbose treatment and the risk of cardiovascular disease and hypertension in patients with impaired glucose tolerance: the STOP-NIDDM trial. *JAMA* 2003; **290**: 486-494

19 **El-Kebbi IM**, Ziemer DC, Cook CB, Gallina DL, Barnes CS, Phillips LS. Utility of casual postprandial glucose levels in type 2 diabetes management. *Diabetes Care* 2004; **27**: 335-339

20 **Monnier L**, Lapinski H, Colette C. Contributions of fasting and postprandial plasma glucose increments to the overall diurnal hyperglycemia of type 2 diabetic patients: variations with increasing levels of HbA(1c). *Diabetes Care* 2003; **26**: 881-885

21 **Bastyr EJ 3rd**, Stuart CA, Brodows RG, Schwartz S, Graf CJ, Zagar A, Robertson KE. Therapy focused on lowering postprandial glucose, not fasting glucose, may be superior for lowering HbA1c. IOEZ Study Group. *Diabetes Care* 2000; **23**: 1236-1241

22 **Haller H**. The clinical importance of postprandial glucose. *Diabetes Res Clin Pract* 1998; **40** Suppl: S43-S49

23 **Ceriello A**. Acute hyperglycaemia and oxidative stress generation. *Diabet Med* 1997; **14** Suppl 3: S45-S49

24 **Ceriello A**. The emerging role of post-prandial hyperglycaemic spikes in the pathogenesis of diabetic complications. *Diabet Med* 1998; **15**: 188-193

25 **Heine RJ**, Dekker JM. Beyond postprandial hyperglycaemia: metabolic factors associated with cardiovascular disease. *Diabetologia* 2002; **45**: 461-475

26 **Horowitz M**, O'Donovan D, Jones KL, Feinle C, Rayner CK, Samsom M. Gastric emptying in diabetes: clinical significance and treatment. *Diabet Med* 2002; **19**: 177-194

27 **Horowitz M,** Edelbroek MA, Wishart JM, Straathof JW. Relationship between oral glucose tolerance and gastric emptying in normal healthy subjects. *Diabetologia* 1993; **36**: 857-862

28 **Jones KL**, Horowitz M, Carney BI, Wishart JM, Guha S, Green L. Gastric emptying in early noninsulin-dependent diabetes mellitus. *J Nucl Med* 1996; **37**: 1643-1648

29 **Gonlachanvit S**, Hsu CW, Boden GH, Knight LC, Maurer AH, Fisher RS, Parkman HP. Effect of altering gastric emptying on postprandial plasma glucose concentrations following a physiologic meal in type-II diabetic patients. *Dig Dis Sci* 2003; **48**: 488-497

30 **Ishii M**, Nakamura T, Kasai F, Onuma T, Baba T, Takebe K. Altered postprandial insulin requirement in IDDM patients with gastroparesis. *Diabetes Care* 1994; **17**: 901-903

31 **Heddle R**, Collins PJ, Dent J, Horowitz M, Read NW, Chatterton B, Houghton LA. Motor mechanisms associated with slowing of the gastric emptying of a solid meal by an intraduodenal lipid infusion. *J Gastroenterol Hepatol* 1989; **4**: 437-447

32 **Heddle R**, Miedema BW, Kelly KA. Integration of canine proximal gastric, antral, pyloric, and proximal duodenal motility during fasting and after a liquid meal. *Dig Dis Sci* 1993; **38**: 856-869

33 **Lin HC**, Doty JE, Reedy TJ, Meyer JH. Inhibition of gastric emptying by glucose depends on length of intestine exposed to nutrient. *Am J Physiol* 1989; **256**: G404-G411

34 **Lin HC**, Doty JE, Reedy TJ, Meyer JH. Inhibition of gastric emptying by sodium oleate depends on length of intestine exposed to nutrient. *Am J Physiol* 1990; **259**: G1031-G1036

35 **Treacy PJ**, Jamieson GG, Dent J, Devitt PG, Heddle R. Duodenal intramural nerves in control of pyloric motility and gastric emptying. *Am J Physiol* 1992; **263**: G1-G5

36 **van Berge Henegouwen MI**, van Gulik TM, Akkermans LM, Jansen JB, Gouma DJ. The effect of octreotide on gastric emptying at a dosage used to prevent complications after pancreatic surgery: a randomised, placebo controlled study in volunteers. *Gut* 1997; **41**: 758-762

37 **Schirra J**, Houck P, Wank U, Arnold R, Göke B, Katschinski M. Effects of glucagon-like peptide-1(7-36)amide on antro-pyloro-duodenal motility in the interdigestive state and with duodenal lipid perfusion in humans. *Gut* 2000; **46**: 622-631

38 **Schirra J**, Nicolaus M, Roggel R, Katschinski M, Storr M, Woerle HJ, Göke B. Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans. *Gut* 2006; **55**: 243-251

39 **Horowitz M**, Nauck MA. To be or not to be--an incretin or enterogastrone? *Gut* 2006; **55**: 148-150

40 **Nauck MA**, Niedereichholz U, Ettler R, Holst JJ, Orskov C, Ritzel R, Schmiegel WH. Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. *Am J Physiol* 1997; **273**: E981-E988

41 **ELRICK H**, STIMMLER L, HLAD CJ, ARAI Y. PLASMA INSULIN RESPONSE TO ORAL AND INTRAVENOUS GLUCOSE ADMINISTRATION. *J Clin Endocrinol Metab* 1964; **24**: 1076-1082

42 **McIntyre N**, Holdsworth CD, Turner DS. Intestinal factors in the control of insulin secretion. *J Clin Endocrinol Metab* 1965; **25**: 1317-1324

43 **Perley MJ**, Kipnis DM. Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic sujbjects. *J Clin Invest* 1967; **46**: 1954-1962

44 **Creutzfeldt W**. The incretin concept today. *Diabetologia* 1979; **16**: 75-85

45 **Holst JJ**, Gromada J. Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic

humans. *Am J Physiol Endocrinol Metab* 2004; **287**: E199-E206
46 **Schirra J**, Katschinski M, Weidmann C, Schäfer T, Wank U, Arnold R, Göke B. Gastric emptying and release of incretin hormones after glucose ingestion in humans. *J Clin Invest* 1996; **97**: 92-103
47 **Meier JJ**, Nauck MA, Schmidt WE, Gallwitz B. Gastric inhibitory polypeptide: the neglected incretin revisited. *Regul Pept* 2002; **107**: 1-13
48 **Toft-Nielsen MB**, Damholt MB, Madsbad S, Hilsted LM, Hughes TE, Michelsen BK, Holst JJ. Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. *J Clin Endocrinol Metab* 2001; **86**: 3717-3723
49 **Horowitz M**, Harding PE, Maddox AF, Wishart JM, Akkermans LM, Chatterton BE, Shearman DJ. Gastric and oesophageal emptying in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1989; **32**: 151-159
50 **O'Donovan D**, Hausken T, Lei Y, Russo A, Keogh J, Horowitz M, Jones KL. Effect of aging on transpyloric flow, gastric emptying, and intragastric distribution in healthy humans--impact on glycemia. *Dig Dis Sci* 2005; **50**: 671-676
51 **de Souza CJ**, Gagen K, Chen W, Dragonas N. Early insulin release effectively improves glucose tolerance: studies in two rodent models of type 2 diabetes mellitus. *Diabetes Obes Metab* 2001; **3**: 85-95
52 **O'Donovan DG**, Doran S, Feinle-Bisset C, Jones KL, Meyer JH, Wishart JM, Morris HA, Horowitz M. Effect of variations in small intestinal glucose delivery on plasma glucose, insulin, and incretin hormones in healthy subjects and type 2 diabetes. *J Clin Endocrinol Metab* 2004; **89**: 3431-3435
53 **Chaikomin R**, Doran S, Jones KL, Feinle-Bisset C, O'Donovan D, Rayner CK, Horowitz M. Initially more rapid small intestinal glucose delivery increases plasma insulin, GIP, and GLP-1 but does not improve overall glycemia in healthy subjects. *Am J Physiol Endocrinol Metab* 2005; **289**: E504-E507
54 **Schwartz MP**, Samsom M, Renooij W, van Steenderen LW, Benninga MA, van Geenen EJ, van Herwaarden MA, de Smet MB, Smout AJ. Small bowel motility affects glucose absorption in a healthy man. *Diabetes Care* 2002; **25**: 1857-1861
55 **HOLDSWORTH CD**, DAWSON AM. THE ABSORPTION OF MONOSACCHARIDES IN MAN. *Clin Sci* 1964; **27**: 371-379
56 **Modigliani R**, Bernier JJ. Absorption of glucose, sodium, and water by the human jejunum studied by intestinal perfusion with a proximal occluding balloon and at variable flow rates. *Gut* 1971; **12**: 184-193
57 **Duchman SM**, Ryan AJ, Schedl HP, Summers RW, Bleiler TL, Gisolfi CV. Upper limit for intestinal absorption of a dilute glucose solution in men at rest. *Med Sci Sports Exerc* 1997; **29**: 482-488
58 **Thomson AB**, Wild G. Adaptation of intestinal nutrient transport in health and disease. Part II. *Dig Dis Sci* 1997; **42**: 470-488
59 **Csáky TZ**, Fischer E. Induction of an intestinal epithelial sugar transport system by high blood sugar. *Experientia* 1977; **33**: 223-224
60 **Csáky TZ**, Fischer E. Intestinal sugar transport in experimental diabetes. *Diabetes* 1981; **30**: 568-574
61 **Fischer E**, Lauterbach F. Effect of hyperglycaemia on sugar transport in the isolated mucosa of guinea-pig small intestine. *J Physiol* 1984; **355**: 567-586
62 **Gottesbüren H**, Schmidt E, Menge H, Bloch R, Lorenz-Meyer H, Riecken EO. The effect of diabetes mellitus and insulin on the absorption of glucose, water and electrolytes by the small intestine in man. *Acta Endocrinol Suppl (Copenh)* 1973; **173**: 130
63 **Costrini NV**, Ganeshappa KP, Wu W, Whalen GE, Soergel KH. Effect of insulin, glucose, and controlled diabetes mellitus on human jejunal function. *Am J Physiol* 1977; **233**: E181-E187
64 **Gulliford MC**, Bicknell EJ, Pover GG, Scarpello JH. Intestinal glucose and amino acid absorption in healthy volunteers and noninsulin-dependent diabetic subjects. *Am J Clin Nutr* 1989; **49**: 1247-1251
65 **Vinnik IE**, Kern F, Sussman KE. The effect of diabetes and insulin on glucose absorption by the small intestine. *J Lab Clin Med* 1967; **66**: 131-136
66 **Dyer J**, Wood IS, Palejwala A, Ellis A, Shirazi-Beechey SP. Expression of monosaccharide transporters in intestine of diabetic humans. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G241-G248
67 **Rayner CK**, Schwartz MP, van Dam PS, Renooij W, de Smet M, Horowitz M, Smout AJ, Samsom M. Small intestinal glucose absorption and duodenal motility in type 1 diabetes mellitus. *Am J Gastroenterol* 2002; **97**: 3123-3130
68 **Chaikomin R**, Wu K-L, Doran S, Smout A, Horowitz M, Rayner C. Evaluation of the effects of hyoscine on duodenal motor function using concurrent multiple intraluminal impedance and manometry (Abstract). *Gastroenterology* 2005; **128**: A672-A673
69 **Eissele R**, Göke R, Willemer S, Harthus HP, Vermeer H, Arnold R, Göke B. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *Eur J Clin Invest* 1992; **22**: 283-291
70 **Holst JJ**. Glucagonlike peptide 1: a newly discovered gastrointestinal hormone. *Gastroenterology* 1994; **107**: 1848-1855
71 **Schirra J**, Kuwert P, Wank U, Leicht P, Arnold R, Göke B, Katschinski M. Differential effects of subcutaneous GLP-1 on gastric emptying, antroduodenal motility, and pancreatic function in men. *Proc Assoc Am Physicians* 1997; **109**: 84-97
72 **Lauritsen KB**, Christensen KC, Stokholm KH. Gastric inhibitory polypeptide (GIP) release and incretin effect after oral glucose in obesity and after jejunoileal bypass. *Scand J Gastroenterol* 1980; **15**: 489-495
73 **Andrews NJ**, Irving MH. Human gut hormone profiles in patients with short bowel syndrome. *Dig Dis Sci* 1992; **37**: 729-732
74 **Mason EE**. Ileal [correction of ilial] transposition and enteroglucagon/GLP-1 in obesity (and diabetic?) surgery. *Obes Surg* 1999; **9**: 223-228
75 **Jeppesen PB**, Hartmann B, Thulesen J, Hansen BS, Holst JJ, Poulsen SS, Mortensen PB. Elevated plasma glucagon-like peptide 1 and 2 concentrations in ileum resected short bowel patients with a preserved colon. *Gut* 2000; **47**: 370-376
76 **Gentilcore D**, Bryant B, Wishart JM, Morris HA, Horowitz M, Jones KL. Acarbose attenuates the hypotensive response to sucrose and slows gastric emptying in the elderly. *Am J Med* 2005; **118**: 1289
77 **Wen J**, Phillips SF, Sarr MG, Kost LJ, Holst JJ. PYY and GLP-1 contribute to feedback inhibition from the canine ileum and colon. *Am J Physiol* 1995; **269**: G945-G952
78 **Horowitz M**, Maddox AF, Wishart JM, Harding PE, Chatterton BE, Shearman DJ. Relationships between oesophageal transit and solid and liquid gastric emptying in diabetes mellitus. *Eur J Nucl Med* 1991; **18**: 229-234
79 **Fraser RJ**, Horowitz M, Maddox AF, Harding PE, Chatterton BE, Dent J. Hyperglycaemia slows gastric emptying in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1990; **33**: 675-680
80 **Schvarcz E**, Palmér M, Aman J, Berne C. Hypoglycemia increases the gastric emptying rate in healthy subjects. *Diabetes Care* 1995; **18**: 674-676
81 **Russo A**, Stevens JE, Chen R, Gentilcore D, Burnet R, Horowitz M, Jones KL. Insulin-induced hypoglycemia accelerates gastric emptying of solids and liquids in long-standing type 1 diabetes. *J Clin Endocrinol Metab* 2005; **90**: 4489-4495
82 **Schvarcz E**, Palmér M, Aman J, Horowitz M, Stridsberg M, Berne C. Physiological hyperglycemia slows gastric emptying in normal subjects and patients with insulin-dependent diabetes mellitus. *Gastroenterology* 1997; **113**: 60-66
83 **Groop LC**, Luzi L, DeFronzo RA, Melander A. Hyperglycaemia and absorption of sulphonylurea drugs. *Lancet* 1989; **2**: 129-130
84 **Horowitz M**, Dent J. Disordered gastric emptying: mechanical basis, assessment and treatment. *Baillieres Clin Gastroenterol* 1991; **5**: 371-407
85 **Sanders KM**. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology* 1996; **111**: 492-515
86 **Hebbard GS**, Samsom M, Sun WM, Dent J, Horowitz M. Hyperglycemia affects proximal gastric motor and sensory function during small intestinal triglyceride infusion. *Am J Physiol* 1996; **271**: G814-G819
87 **Hebbard GS**, Sun WM, Dent J, Horowitz M. Hyperglycaemia affects proximal gastric motor and sensory function in normal subjects. *Eur J Gastroenterol Hepatol* 1996; **8**: 211-217

88 **Rayner CK**, Verhagen MA, Hebbard GS, DiMatteo AC, Doran SM, Horowitz M. Proximal gastric compliance and perception of distension in type 1 diabetes mellitus: effects of hyperglycemia. *Am J Gastroenterol* 2000; **95**: 1175-1183
89 **Barnett JL**, Owyang C. Serum glucose concentration as a modulator of interdigestive gastric motility. *Gastroenterology* 1988; **94**: 739-744
90 **Björnsson ES**, Urbanavicius V, Eliasson B, Attvall S, Smith U, Abrahamsson H. Effects of hyperglycemia on interdigestive gastrointestinal motility in humans. *Scand J Gastroenterol* 1994; **29**: 1096-1104
91 **Hasler WL**, Soudah HC, Dulai G, Owyang C. Mediation of hyperglycemia-evoked gastric slow-wave dysrhythmias by endogenous prostaglandins. *Gastroenterology* 1995; **108**: 727-736
92 **Samsom M**, Smout AJ. Abnormal gastric and small intestinal motor function in diabetes mellitus. *Dig Dis* 1997; **15**: 263-274
93 **Fraser R**, Horowitz M, Dent J. Hyperglycaemia stimulates pyloric motility in normal subjects. *Gut* 1991; **32**: 475-478
94 **Hebbard GS**, Samson M, Andrews JM, Carman D, Tansell B, Sun WM, Dent J, Horowitz M. Hyperglycemia affects gastric electrical rhythm and nausea during intraduodenal triglyceride infusion. *Dig Dis Sci* 1997; **42**: 568-575
95 **Verhagen MA**, Rayner CK, Andrews JM, Hebbard GS, Doran SM, Samsom M, Horowitz M. Physiological changes in blood glucose do not affect gastric compliance and perception in normal subjects. *Am J Physiol* 1999; **276**: G761-G766
96 **Jones KL**, Berry M, Kong MF, Kwiatek MA, Samsom M, Horowitz M. Hyperglycemia attenuates the gastrokinetic effect of erythromycin and affects the perception of postprandial hunger in normal subjects. *Diabetes Care* 1999; **22**: 339-344
97 **Petrakis IE**, Vrachassotakis N, Sciacca V, Vassilakis SI, Chalkiadakis G. Hyperglycaemia attenuates erythromycin-induced acceleration of solid-phase gastric emptying in idiopathic and diabetic gastroparesis. *Scand J Gastroenterol* 1999; **34**: 396-403
98 **Rayner CK**, Su YC, Doran SM, Jones KL, Malbert CH, Horowitz M. The stimulation of antral motility by erythromycin is attenuated by hyperglycemia. *Am J Gastroenterol* 2000; **95**: 2233-2241
99 **Lingenfelser T**, Sun W, Hebbard GS, Dent J, Horowitz M. Effects of duodenal distension on antropyloroduodenal pressures and perception are modified by hyperglycemia. *Am J Physiol* 1999; **276**: G711-G718
100 **Oster-Jørgensen E**, Qvist N, Pedersen SA, Rasmussen L, Hovendal CP. The influence of induced hyperglycaemia on the characteristics of intestinal motility and bile kinetics in healthy men. *Scand J Gastroenterol* 1992; **27**: 285-288
101 **Russo A**, Fraser R, Horowitz M. The effect of acute hyperglycaemia on small intestinal motility in normal subjects. *Diabetologia* 1996; **39**: 984-989
102 **Virally-Monod M**, Tielmans D, Kevorkian JP, Bouhnik Y, Flourie B, Porokhov B, Ajzenberg C, Warnet A, Guillausseau PJ. Chronic diarrhoea and diabetes mellitus: prevalence of small intestinal bacterial overgrowth. *Diabetes Metab* 1998; **24**: 530-536
103 **Samsom M**, Akkermans LM, Jebbink RJ, van Isselt H, vanBerge-Henegouwen GP, Smout AJ. Gastrointestinal motor mechanisms in hyperglycaemia induced delayed gastric emptying in type I diabetes mellitus. *Gut* 1997; **40**: 641-646
104 **Rayner CK**, Horowitz M. Gastrointestinal motility and glycemic control in diabetes: the chicken and the egg revisited? *J Clin Invest* 2006; **116**: 299-302
105 **Fregonesi CE**, Miranda-Neto MH, Molinari SL, Zanoni JN. Quantitative study of the myenteric plexus of the stomach of rats with streptozotocin-induced diabetes. *Arq Neuropsiquiatr* 2001; **59**: 50-53
106 **Watkins CC**, Sawa A, Jaffrey S, Blackshaw S, Barrow RK, Snyder SH, Ferris CD. Insulin restores neuronal nitric oxide synthase expression and function that is lost in diabetic gastropathy. *J Clin Invest* 2000; **106**: 373-384
107 **Ordög T**, Takayama I, Cheung WK, Ward SM, Sanders KM. Remodeling of networks of interstitial cells of Cajal in a murine model of diabetic gastroparesis. *Diabetes* 2000; **49**: 1731-1739
108 **Forster J**, Damjanov I, Lin Z, Sarosiek I, Wetzel P, McCallum RW. Absence of the interstitial cells of Cajal in patients with gastroparesis and correlation with clinical findings. *J Gastrointest Surg* 2005; **9**: 102-108
109 **Anitha M**, Gondha C, Sutliff R, Parsadanian A, Mwangi S, Sitaraman SV, Srinivasan S. GDNF rescues hyperglycemia-induced diabetic enteric neuropathy through activation of the PI3K/Akt pathway. *J Clin Invest* 2006; **116**: 344-356
110 **Liu M**, Seino S, Kirchgessner AL. Identification and characterization of glucoresponsive neurons in the enteric nervous system. *J Neurosci* 1999; **19**: 10305-10317
111 **Lam WF**, Masclee AA, de Boer SY, Lamers CB. Hyperglycemia reduces gastric secretory and plasma pancreatic polypeptide responses to modified sham feeding in humans. *Digestion* 1993; **54**: 48-53
112 **Yeap BB**, Russo A, Fraser RJ, Wittert GA, Horowitz M. Hyperglycemia affects cardiovascular autonomic nerve function in normal subjects. *Diabetes Care* 1996; **19**: 880-882
113 **Ceriello A**, Hanefeld M, Leiter L, Monnier L, Moses A, Owens D, Tajima N, Tuomilehto J. Postprandial glucose regulation and diabetic complications. *Arch Intern Med* 2004; **164**: 2090-2095
114 **Hassan Y**, Johnson B, Nader N, Gannon MC, Nuttall FQ. The relationship between 24-hour integrated glucose concentrations and % glycohemoglobin. *J Lab Clin Med* 2006; **147**: 21-26
115 **Ishii M**, Nakamura T, Kasai F, Baba T, Takebe K. Erythromycin derivative improves gastric emptying and insulin requirement in diabetic patients with gastroparesis. *Diabetes Care* 1997; **20**: 1134-1137
116 **Westman EC**, Yancy WS Jr, Humphreys M. Dietary treatment of diabetes mellitus in the pre-insulin era (1914-1922). *Perspect Biol Med* 2006; **49**: 77-83
117 **Liu S**, Willett WC, Stampfer MJ, Hu FB, Franz M, Sampson L, Hennekens CH, Manson JE. A prospective study of dietary glycemic load, carbohydrate intake, and risk of coronary heart disease in US women. *Am J Clin Nutr* 2000; **71**: 1455-1461
118 **Boden G**, Sargrad K, Homko C, Mozzoli M, Stein TP. Effect of a low-carbohydrate diet on appetite, blood glucose levels, and insulin resistance in obese patients with type 2 diabetes. *Ann Intern Med* 2005; **142**: 403-411
119 **Gutierrez M**, Akhavan M, Jovanovic L, Peterson CM. Utility of a short-term 25% carbohydrate diet on improving glycemic control in type 2 diabetes mellitus. *J Am Coll Nutr* 1998; **17**: 595-600
120 **Farnsworth E**, Luscombe ND, Noakes M, Wittert G, Argyiou E, Clifton PM. Effect of a high-protein, energy-restricted diet on body composition, glycemic control, and lipid concentrations in overweight and obese hyperinsulinemic men and women. *Am J Clin Nutr* 2003; **78**: 31-39
121 **Samaha FF**, Iqbal N, Seshadri P, Chicano KL, Daily DA, McGrory J, Williams T, Williams M, Gracely EJ, Stern L. A low-carbohydrate as compared with a low-fat diet in severe obesity. *N Engl J Med* 2003; **348**: 2074-2081
122 **Stern L**, Iqbal N, Seshadri P, Chicano KL, Daily DA, McGrory J, Williams M, Gracely EJ, Samaha FF. The effects of low-carbohydrate versus conventional weight loss diets in severely obese adults: one-year follow-up of a randomized trial. *Ann Intern Med* 2004; **140**: 778-785
123 **Gannon MC**, Nuttall FQ, Neil BJ, Westphal SA. The insulin and glucose responses to meals of glucose plus various proteins in type II diabetic subjects. *Metabolism* 1988; **37**: 1081-1088
124 **Gannon MC**, Nuttall FQ, Saeed A, Jordan K, Hoover H. An increase in dietary protein improves the blood glucose response in persons with type 2 diabetes. *Am J Clin Nutr* 2003; **78**: 734-741
125 **Wolever TM**. The glycemic index. *World Rev Nutr Diet* 1990; **62**: 120-185
126 **Ludwig DS**. The glycemic index: physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. *JAMA* 2002; **287**: 2414-2423
127 **Björck I**, Elmståhl HL. The glycaemic index: importance of dietary fibre and other food properties. *Proc Nutr Soc* 2003; **62**: 201-206
128 **Mourot J**, Thouvenot P, Couet C, Antoine JM, Krobicka A, Debry G. Relationship between the rate of gastric emptying and glucose and insulin responses to starchy foods in young

healthy adults. *Am J Clin Nutr* 1988; **48**: 1035-1040
129 **Hallfrisch J**, Facn KM. Mechanisms of the effects of grains on insulin and glucose responses. *J Am Coll Nutr* 2000; **19**: 320S-325S
130 **Wolever TM**. Relationship between dietary fiber content and composition in foods and the glycemic index. *Am J Clin Nutr* 1990; **51**: 72-75
131 **Benini L**, Castellani G, Brighenti F, Heaton KW, Brentegani MT, Casiraghi MC, Sembenini C, Pellegrini N, Fioretta A, Minniti G. Gastric emptying of a solid meal is accelerated by the removal of dietary fibre naturally present in food. *Gut* 1995; **36**: 825-830
132 **Cherbut C**, Bruley des Varannes S, Schnee M, Rival M, Galmiche JP, Delort-Laval J. Involvement of small intestinal motility in blood glucose response to dietary fibre in man. *Br J Nutr* 1994; **71**: 675-685
133 **Leclère CJ**, Champ M, Boillot J, Guille G, Lecannu G, Molis C, Bornet F, Krempf M, Delort-Laval J, Galmiche JP. Role of viscous guar gums in lowering the glycemic response after a solid meal. *Am J Clin Nutr* 1994; **59**: 914-921
134 **Jones KL**, MacIntosh C, Su YC, Wells F, Chapman IM, Tonkin A, Horowitz M. Guar gum reduces postprandial hypotension in older people. *J Am Geriatr Soc* 2001; **49**: 162-167
135 **Blackburn NA**, Redfern JS, Jarjis H, Holgate AM, Hanning I, Scarpello JH, Johnson IT, Read NW. The mechanism of action of guar gum in improving glucose tolerance in man. *Clin Sci* (Lond) 1984; **66**: 329-336
136 **O'Donovan D**, Feinle-Bisset C, Chong C, Cameron A, Tonkin A, Wishart J, Horowitz M, Jones KL. Intraduodenal guar attenuates the fall in blood pressure induced by glucose in healthy older adults. *J Gerontol A Biol Sci Med Sci* 2005; **60**: 940-946
137 **Nilsson M**, Stenberg M, Frid AH, Holst JJ, Björck IM. Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins. *Am J Clin Nutr* 2004; **80**: 1246-1253
138 **Anderson GH**, Woodend D. Effect of glycemic carbohydrates on short-term satiety and food intake. *Nutr Rev* 2003; **61**: S17-S26
139 **Roberts SB**. Glycemic index and satiety. *Nutr Clin Care* 2003; **6**: 20-26
140 **Uusitupa MI**. Fructose in the diabetic diet. *Am J Clin Nutr* 1994; **59**: 753S-757S
141 **Vozzo R**, Baker B, Wittert GA, Wishart JM, Morris H, Horowitz M, Chapman I. Glycemic, hormone, and appetite responses to monosaccharide ingestion in patients with type 2 diabetes. *Metabolism* 2002; **51**: 949-957
142 **Rayner CK**, Park HS, Wishart JM, Kong M, Doran SM, Horowitz M. Effects of intraduodenal glucose and fructose on antropyloric motility and appetite in healthy humans. *Am J Physiol Regul Integr Comp Physiol* 2000; **278**: R360-R366
143 **Miller JC**. Importance of glycemic index in diabetes. *Am J Clin Nutr* 1994; **59**: 747S-752S
144 **Heilbronn LK**, Noakes M, Clifton PM. The effect of high- and low-glycemic index energy restricted diets on plasma lipid and glucose profiles in type 2 diabetic subjects with varying glycemic control. *J Am Coll Nutr* 2002; **21**: 120-127
145 **Brand-Miller J**, Hayne S, Petocz P, Colagiuri S. Low-glycemic index diets in the management of diabetes: a meta-analysis of randomized controlled trials. *Diabetes Care* 2003; **26**: 2261-2267
146 **Schwartz JG**, Guan D, Green GM, Phillips WT. Treatment with an oral proteinase inhibitor slows gastric emptying and acutely reduces glucose and insulin levels after a liquid meal in type II diabetic patients. *Diabetes Car*e 1994; **17**: 255-262
147 **Berry MK**, Russo A, Wishart JM, Tonkin A, Horowitz M, Jones KL. Effect of solid meal on gastric emptying of, and glycemic and cardiovascular responses to, liquid glucose in older subjects. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G655-G662
148 **Horowitz M**, Jones K, Edelbroek MA, Smout AJ, Read NW. The effect of posture on gastric emptying and intragastric distribution of oil and aqueous meal components and appetite. *Gastroenterology* 1993; **105**: 382-390
149 **Cunningham KM**, Read NW. The effect of incorporating fat into different components of a meal on gastric emptying and postprandial blood glucose and insulin responses. *Br J Nutr* 1989; **61**: 285-290
150 **Gentilcore D**, Chaikomin R, Jones KL, Russo A, Feinle-Bisset C, Wishart JM, Rayner CK, Horowitz M. Effects of fat on gastric emptying of and the glycemic, insulin, and incretin responses to a carbohydrate meal in type 2 diabetes. *J Clin Endocrinol Metab* 2006; **91**: 2062-2067
151 **Samsom M**, Szarka LA, Camilleri M, Vella A, Zinsmeister AR, Rizza RA. Pramlintide, an amylin analog, selectively delays gastric emptying: potential role of vagal inhibition. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G946-G951
152 **Thompson RG**, Pearson L, Kolterman OG. Effects of 4 weeks' administration of pramlintide, a human amylin analogue, on glycaemia control in patients with IDDM: effects on plasma glucose profiles and serum fructosamine concentrations. *Diabetologia* 1997; **40**: 1278-1285
153 **Thompson RG**, Pearson L, Schoenfeld SL, Kolterman OG. Pramlintide, a synthetic analog of human amylin, improves the metabolic profile of patients with type 2 diabetes using insulin. The Pramlintide in Type 2 Diabetes Group. *Diabetes Care* 1998; **21**: 987-993
154 **Ratner RE**, Dickey R, Fineman M, Maggs DG, Shen L, Strobel SA, Weyer C, Kolterman OG. Amylin replacement with pramlintide as an adjunct to insulin therapy improves long-term glycaemic and weight control in Type 1 diabetes mellitus: a 1-year, randomized controlled trial. *Diabet Med* 2004; **21**: 1204-1212
155 **Hollander PA**, Levy P, Fineman MS, Maggs DG, Shen LZ, Strobel SA, Weyer C, Kolterman OG. Pramlintide as an adjunct to insulin therapy improves long-term glycemic and weight control in patients with type 2 diabetes: a 1-year randomized controlled trial. *Diabetes Care* 2003; **26**: 784-790
156 **Chapman I**, Parker B, Doran S, Feinle-Bisset C, Wishart J, Strobel S, Wang Y, Burns C, Lush C, Weyer C, Horowitz M. Effect of pramlintide on satiety and food intake in obese subjects and subjects with type 2 diabetes. *Diabetologia* 2005; **48**: 838-848
157 **Bischoff H**. Pharmacology of alpha-glucosidase inhibition. *Eur J Clin Invest* 1994; **24** Suppl 3: 3-10
158 **Seifarth C**, Bergmann J, Holst JJ, Ritzel R, Schmiegel W, Nauck MA. Prolonged and enhanced secretion of glucagon-like peptide 1 (7-36 amide) after oral sucrose due to alpha-glucosidase inhibition (acarbose) in Type 2 diabetic patients. *Diabet Med* 1998; **15**: 485-491
159 **Ranganath L**, Norris F, Morgan L, Wright J, Marks V. Delayed gastric emptying occurs following acarbose administration and is a further mechanism for its anti-hyperglycaemic effect. *Diabet Med* 1998; **15**: 120-124
160 **Hücking K**, Kostic Z, Pox C, Ritzel R, Holst JJ, Schmiegel W, Nauck MA. alpha-Glucosidase inhibition (acarbose) fails to enhance secretion of glucagon-like peptide 1 (7-36 amide) and to delay gastric emptying in Type 2 diabetic patients. *Diabet Med* 2005; **22**: 470-476
161 **Hirsh AJ**, Yao SY, Young JD, Cheeseman CI. Inhibition of glucose absorption in the rat jejunum: a novel action of alpha-D-glucosidase inhibitors. *Gastroenterology* 1997; **113**: 205-211
162 **Valverde I**, Puente J, Martín-Duce A, Molina L, Lozano O, Sancho V, Malaisse WJ, Villanueva-Peñacarrillo ML. Changes in glucagon-like peptide-1 (GLP-1) secretion after biliopancreatic diversion or vertical banded gastroplasty in obese subjects. *Obes Surg* 2005; **15**: 387-397
163 **Fineman MS**, Bicsak TA, Shen LZ, Taylor K, Gaines E, Varns A, Kim D, Baron AD. Effect on glycemic control of exenatide (synthetic exendin-4) additive to existing metformin and/or sulfonylurea treatment in patients with type 2 diabetes. *Diabetes Care* 2003; **26**: 2370-2377
164 **Holst JJ**. Treatment of type 2 diabetes mellitus with agonists of the GLP-1 receptor or DPP-IV inhibitors. *Expert Opin Emerg Drugs* 2004; **9**: 155-166

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REVIEW

Assessing risks for gastric cancer: New tools for pathologists

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Abstract

Although the Sydney Systems (original and updated) for the classification of gastritis have contributed substantially to the uniformity of the reporting of gastric conditions, they lack immediacy in conveying to the user information about gastric cancer risk. In this review, we summarize the current understanding of the gastric lesions associated with an increased risk for cancer, and present the rationale for a proposal for new ways of reporting gastritis. In addition to the traditional histopathological data gathered and evaluated according to the Sydney System rules, pathologists could add an assessment expressed as grading and staging of the gastric inflammatory and atrophic lesions and integrate these findings with pertinent laboratory information on pepsinogens and gastrin levels. Such an integrated report could facilitate clinicians' approach to the management of patients with gastric conditions.

Key words: Gastritis; Staging and grading; Gastritis; Histology

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INTRODUCTION

Chronic gastritis is an inflammatory condition of the gastric mucosa characterized by elementary lesions whose type, extent, and distribution are related to their etiology and modulated by host responses and environmental factors[1]. Infection with *H pylori*, which affects an estimated three to four billion persons worldwide, is by far the most common cause of chronic active gastritis; chemical agents, autoimmune phenomena, and other infections account for a very small proportion of chronic, usually non-active gastritides. *H pylori*-gastritis is epidemiologically and biologically linked to the development of gastric cancer[2] and *H pylori* has been listed as a class I carcinogen[3]. Epidemiological and pathological data suggest that extent, intensity, and distribution patterns of gastric inflammation and atrophy are consistently related to the incidence of gastric cancer in a population[4-7]. Although odd-ratios for gastric cancer and peptic ulcer risk in relationship with the type of gastritis have been estimated, most often retrospectively, only in small series and in few populations[5,8-10], it is widely accepted that the accurate histopathological assessment of the gastric mucosa could serve as a reasonably good predictor of cancer risk in an individual patient. In fact, most recent classifications of gastritis have contained the implicit aim of providing a clinico-pathological correlation that could be both synchronous (that is, at the time of the sampling) and, more usefully, diachronic.

When appropriate sampling is available, the histopathological features of the gastric mucosa recognized as being part of the neoplastic process and broadly referred to as "pre-neoplastic lesions" (atrophy, pyloric and intestinal metaplasia, epithelial dysplasia) can be accurately evaluated by the microscopic examination of mucosal biopsies. Although classification systems such as the Sydney System[11], its Houston-updated version[12], and the more recent guidelines for the evaluation of atrophy[13] suggest strategies for the formulation of histopathological reports, we still lack a way to translate the pathological information into a standardized report that would convey comprehensive information on the gastric condition while lending itself to a straightforward analysis of cancer risk.

The purpose of this article is to explore ways for pathologists to maximize the predictive value of the gastric evaluation by: (1) streamlining the histopathological report of gastric biopsies, and (2) integrating relevant laboratory information with pathological data.

GASTRIC MUCOSAL CHANGES RELATED TO GASTRIC CANCER

As a result of seminal field studies conducted by Max Siurala in Finland and Estonia[14-17] and Pelayo Correa in Colombia[4,18-19], as well as the crucial body of knowledge derived from decades of Japanese studies[20,21], the separate entities of chronic superficial gastritis, atrophy, metaplasia, dysplasia and carcinoma were integrated into a hypothetical

sequence known as Correa's cascade[22]. Increasingly well-documented by patho-epidemiological studies, the 1984 multi-step hypothesis of gastric carcinogenesis still lacked an etiological initiator. The missing first step was discovered in the same year[23] and *H pylori* found its place at the top of the cascade[24].

The histopathological lesions broadly regarded as preneoplastic are chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and neoplasia. Their evolution in a cohort can be viewed as a pyramid with a very large base representing the entire *H pylori*-infected population; a segment of these subjects (generally larger in developing than in industrialized areas) will progress to atrophic gastritis, mostly accompanied by intestinal metaplasia. A very small minority will progress further to dysplasia with some eventually developing adenocarcinoma. The closer a lesion is to neoplasia, the more likely it will progress into it. Thus, whereas chronic gastritis is a remote and uncertain precursor of gastric cancer that could be better called a "predisposing condition," high-grade dysplasia is considered already a neoplastic lesion[25,26]. If pathologists could make a reliable assessment of the risk that each patient has, based on a staging of the disease, effective strategies could be developed to detect the early, curable phase of gastric cancer and prevent its progression.

Chronic gastritis

The risk of gastric cancer for a patient with simple, non-atrophic *H pylori* gastritis is negligible, thus, the decision to treat the infection is based, in most cases, on other considerations. There is, however, one exception. Gastric cancer and atrophic gastritis associated with it have at least some familial predisposition[27-30]; therefore, it would seem wise to treat *H pylori* infection as early as possible in direct relatives of patients with gastric cancer. This is one of the rare circumstances in which *H pylori* would be eradicated for the specific purpose of preventing gastric carcinoma in an individual patient.

Atrophy

Gastric atrophy is defined as the loss of appropriate glands in a given gastric compartment[13,31]. This purely histopathological definition indicates that the glands expected to be present in the portion of gastric mucosa under examination (for example, oxyntic glands in the mucosa of the corpus) are no longer there, and have been replaced by something else that does not belong to that area. This "something else" may be extracellular matrix, fibroblasts and collagen, or other glands that normally are not there (e.g., intestinal-type glands or pseudo-pyloric glands). Any of these replacements prevents that portion of gastric mucosa from performing its normal functions (e.g., to secrete acid). Thus, the functional correlate of atrophy is strictly related to its extension.

Atrophic gastritis is a condition characterized by the presence of significant areas of atrophy. Its two most common underlying causes are chronic infection with *H pylori* and the autoimmune gastritis that may become associated with pernicious anemia. In the Updated Sydney System, the term "atrophic gastritis" is used in contrast to "non-atrophic gastritis" or simply "gastritis," a condition usually more severe in the antrum (hence the term "antral-predominant") found in most subjects infected with *H pylori* in the Western industrialized world.

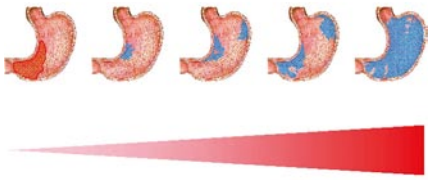

Figure 1 Schematic representation of the progression of atrophy, from absent in the case of antrum-predominant non-atrophic gastritis depicted on the left to the almost generalized metaplastic atrophy depicted on the right. The increased extension of atrophy corresponds to an increased cancer risk, indicated as an expanding triangle. The extension of atrophy can also be reported as a stage from 0 to 4.

The stomach affected by atrophic gastritis shows a decrease or absence of appropriate glands, an expansion of the antral-type mucosa into the corpus ("antralization" or pseudo-pyloric metaplasia) and usually extensive areas of intestinal metaplasia. This condition has been known for several decades to represent a significant epidemiological risk factor for gastric adenocarcinoma[14,17,24,32-36]; as schematically depicted in Figure 1, its prognostic implications in the individual patient seem to be related to the extent and distribution of the atrophic areas[10,37].

Intestinal metaplasia

Intestinal metaplasia is the replacement of the normal gastric mucosa with an epithelium similar to that of the intestine. Attempts to classify the different types of intestinal metaplasia have resulted in a confusing terminology (complete *vs* incomplete, type 1, 2a and 2b, *etc.*); the classification currently used was proposed by Jass and Filipe[38,39]: Type I (brush border and no sulfomucins); Type II (no brush border, rare sulfomucins); and Type III (no brush border, cellular disarray, abundant sulfomucins). Type I intestinal metaplasia has been often said to pose little increased risk of developing carcinoma, whereas type III has been considered as an already dysplastic lesion[10,40-42]. The classification of the three types of metaplasia requires relatively sophisticated histochemical techniques and is far from being standardized. Furthermore, the data suggesting different cancer risks for the different types of intestinal metaplasia are not unequivocal[43]. Therefore, immunohistochemical sub-typing of intestinal metaplasia should be limited to the clinical research setting and not a part of the routine evaluation of patients with intestinal metaplasia.

Dysplasia

Malignancy is the final step of progressive genetic and phenotypic changes that modify the original cellular morphology, eventually generating a biologically new cell characterized by uncontrolled growth and the potential to migrate and implant in locations beyond its original fixed

site. This biological process has been called multi-step or step-wise oncogenesis. In epithelial tissues (for example, the squamous lining of the uterine cervix or the columnar lining of the colon) the first of step visible to an observer using a light microscope is a change in the morphology of the cells that form the epithelium. Nuclei are larger, nucleoli may be prominent and the chromatin may be clumped or granular; compared to the larger nucleus, the cytoplasm appear smaller, a phenomenon referred to as "increased nucleo-cytoplasmic ratio". Various degrees of disarray of the orderly structure of the normal epithelium usually accompanying these changes. Epithelial alterations of this kind occur in two situations: when the epithelium has been injured and is undergoing repair, and when genetic alterations have transformed the cells in a neoplastic growth. It is generally agreed by pathologists that in the former instance one refers to the phenomenon as "regenerative atypia", whereas in the latter case the term "dysplasia" is used[44].

The importance of recognizing and correctly identifying dysplasia is self-evident: while regenerative atypia is the desired response to epithelial injury and an essential part of an organism's homeostasis, dysplasia is the harbinger of cancer and requires immediate action. However, the morphological differences between atypia and dysplasia are not always apparent, and significant areas of phenotypic overlap exist between the two. Pathologists have tried for years to standardize the criteria for the diagnosis and grading of dysplasia in tissues accessible to biopsy sampling. Without getting into the complex historical details of the process, for the purpose of this review we say only that, through the efforts of pioneers such as the late Rodger Haggitt, Robert Riddell, Brian Reid, and others, a satisfactory level of agreement has been reached for dysplasia of the colon and of Barrett's epithelium[44-46]. Gastric dysplasia has received less attention in the past, with only one major consensus article addressing the issue before 1996[47].

In the last decade, the discovery of *H pylori* and its relationship with gastric cancer has stimulated increasing attention to the preneoplastic lesions of the stomach. The possibility that curing this infection could prevent or even cause the regression of such lesions has highlighted the need for uniform and rigorous definitions and diagnostic criteria. However, unlike metaplasia, whose recognition has always been largely free of major disputes, or atrophy, which has been the focus of major conceptual disagreements among pathologists, dysplasia exposed a novel angle of controversy: a pathological schism between East and West, or, more accurately, between Japan and the West[48].

Japan is one of the countries with the highest incidence of adenocarcinoma of the stomach in the world; at the same time, it also has the world's best survival rates for gastric cancer. Although the effective early detection programs, innovative endoscopic techniques, and daring and successful therapeutic endoscopists have been invoked to explain the Japanese success in this area, another explanation has been suggested, mostly in a veiled or oblique manner. To state it simply, it has been implied that, to have such good survival rates the Japanese must call cancer what others call dysplasia. The question has been propelled into the international scientific forum only recently, through the efforts of RJ Schlemper, who in 1996 organized a workshop to address the issue. This workshop resulted in a seminal paper entitled "Differences in diagnostic criteria for gastric carcinoma between Japanese and Western pathologists," published in the Lancet in 1997[49]. Following the workshop and publication of its findings, several other groups have formed to tackle the problem in the traditional pathologists' fashion: by trying to measure the level of agreement (or disagreement) amongst observers. These groups included various proportions of Japanese and Western pathologists, and the ultimate aim was to reach a consensus that classification, if used globally, would allow comparative studies performed in different countries. As a result, new issues have emerged and new classifications have been proposed. The classification currently accepted by the World Health Organization[50] is largely modeled on the consensus agreement reached in Padua, Italy, in 1998[26], and summarizes one of the most recent proposals for an integrated therapeutic and pathological approach[51].

The Padova model is based: (1) on the definition of dysplasia as pre-invasive neoplasia; and (2) on a five-category classification of gastric neoplasia which includes: 1, negative for dysplasia; 2, indefinite for dysplasia; 3, non-invasive neoplasia; 4, suspicious for invasive cancer; 5, gastric cancer. The numerical prefix assigned to each diagnostic category essentially corresponds to the diagnostic categories of the Japanese Classification for Gastric Cancer[52]. Within each category one or more sub-categories are hierarchically ordered to cover the spectrum of epithelial alterations.

THE IMPORTANCE OF GOOD SAMPLING

The topographic distribution of inflammatory infiltrates, lymphoid follicles, atrophy, and metaplasia is an essential determinant used for all classifications of gastritis. These changes may be patchily distributed and their relative intensity in different parts of the stomach may be highly variable. Furthermore, the inflammatory and atrophic processes have different phenotypical expressions in different regions of the stomach. Therefore, to obtain an accurate picture of gastritis, pathologists must have a set of specimens representative of each gastric compartment. Each specimen is examined according to uniform criteria, a general impression of the intensity of the features of gastritis is extrapolated from the various specimens from each compartment, and finally this information is amalgamated in a topographical diagnosis. The location of the biopsy specimens recommended by the Updated Sydney System[12] is depicted in the left panel of Figure 2. A suggestion has been made to replace the original sites with others, purportedly more likely to yield information about the extension of intestinal metaplasia[53], but in the absence of independent testing no proposal in this sense has been presented.

Irrespective of the protocol used, gastroenterologists must keep in mind that the predictive information they can get from their pathologist is only as good as the

biopsy sampling submitted for examination. The Sydney System 5-biopsy protocol is a compromise between what is practically doable in routine practice and the ideal need for maximal topographic information. As depicted in Figure 2, right panel, in special situations such as the diagnosis and follow-up of gastric mucosa-associated lymphoid tissue B-cell lymphomas or the diachronic investigation of dysplasia much more extensive sampling protocols need to be applied[48,54-56].

VIRTUAL HISTOPATHOLOGY

The determination of serum pepsinogens Ⅰ (PG Ⅰ) and Ⅱ (PG Ⅱ), gastrin-17 (G-17) and IgG anti-*H pylori* antibodies by ELISA has been proposed as an array of non-invasive markers for the assessment of both morphological and functional status of the gastric mucosa[57]. The rationale for this approach, described by its enthusiastic supporters as the "serological biopsy," rests on the fact that PG Ⅰ is exclusively secreted by oxyntic glands and represents an excellent marker of the secretory ability of the gastric corpus. In contrast, PG Ⅱ is produced by all types of gastric and duodenal glands and its production is influenced by gastric inflammation[58,59]. Although these molecules are secreted into the gastric lumen, small amounts seep out into the bloodstream and can be measured. Gastrin-17 (G-17), produced in the antrum and secreted directly into the blood, is a specific marker of G cell function[60]. Several studies have now shown that serum levels of PG Ⅰ, PG Ⅱ and G-17 are high in subjects with *H pylori* non-atrophic chronic gastritis[61]. Both PG Ⅰ and PG Ⅱ concentrations are found to decrease significantly two months after the eradication of *H pylori*[62,63]. Furthermore, the ratio of PG Ⅰ and G-17 levels have been found to correlate well with the histopathological diagnosis of atrophic body gastritis and, in some studies, to be associated with the presence of gastric cancer[64-66].

In a recent study, De Mario and his colleagues[67] demonstrated that the analysis of serum pepsinogens, G-17 and anti-*H pylori* IgG levels provide consistent and reproducible information regarding gastric atrophy and its association with *H pylori*. The authors suggest that dyspeptic patients with normal PG Ⅰ, PG Ⅱ, G-17 and a negative serological test for *H pylori* can be reassured that they are unlikely to have peptic ulcer disease and can be treated symptomatically. In contrast, patients with panel test results indicating *H pylori*-related chronic gastritis, with or without atrophy, could either be treated for *H pylori* or referred for endoscopy, depending on the type and severity of their manifestations.

GENERATING A CLINICALLY USEFUL HISTOPATHOLOGY REPORT

The article reporting the Updated Sydney System, published in October 1996, has recently passed the 1000-citation milestone[68], suggesting that the semi-quantitative scoring system it advocated remains a useful tool for clinical research. Nevertheless, the same pathologists who use it when assessing biopsies for clinical studies find it too cumbersome to use in their routine diagnostic activities.

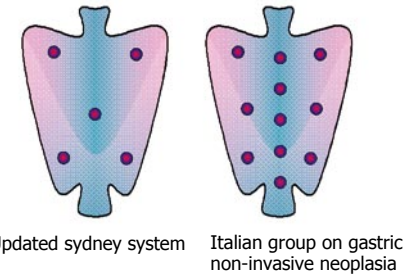

Figure 2 Two different biopsy protocols.

Using the framework provided by the Sydney System's and the Atrophy Club's analytic approach, we have recently put forward a proposal for a grading and staging scheme that integrates the relevant histopathological data gathered and interpreted by the pathologist and delivers them in the form of a simple, yet information-rich report[69]. We have suggested that the method is both feasible and practical, and that staging and grading (preceded by a description of the histological findings in the biopsy samples) could represent the concluding message of the histological report. This scheme could be do for chronic gastritis what the grading and staging system introduced by the International Group of Hepatologists in 1995 did for chronic hepatitis: make prognostically significant and reproducible information immediately available in the clinical practice[70,71].

Briefly, the proposal consists of summarizing the combined intensity of mononuclear and scoring granulocytic inflammation in both antral and oxyntic biopsy samples in a grade from 0 (no inflammation) to 4 (a very dense infiltrate in all the biopsy samples). The extent of atrophy, with or without intestinal metaplasia, would be reported as a stage from 0 (no atrophy) to 4 (pan-atrophy involving all antral and oxyntic samples). The latter would convey information on the anatomical extent of the atrophic-metaplastic changes related to cancer risk. Figure 1 shows the progression from stage 0 (left) to stage 4 (right).

A pathologist who had access to the results of the "serological biopsy" and applied the grading and staging principles outlined in this scheme could generate a comprehensive informative integrated report that could be used by clinicians as a solid base for the management of patients with gastric conditions.

This proposal has been discussed at an international consensus group of gastroenterologists and pathologists (Operative Link for Gastritis Assessment-OLGA) that gathered in Parma, Italy, in April 2005. The group included Massimo Rugge, Padova, Italy; Pelayo Correa, New Orleans, Louisiana, USA; Francesco Di Mario, Parma, Italy; Emad El-Omar, Aberdeen, Scotland, UK; Roberto Fiocca, Genova, Italy; Karel Geboes, Leuven, Belgium; David Y Graham, Houston, Texas, USA; Takanori Hattori, Shiga, Japan; Peter Malfertheiner, Magdeburg, Germany; Pentti Sipponen, Espoo, Finland; Joseph Sung, Hong Kong, China; Wilfred Weinstein, Los Angeles, California,

USA; Michael Vieth, Bayreuth, Germany; and Robert M Genta, Geneva, Switzerland.

After deliberations that led to a number of modifications of the original proposal, the OLGA group has agreed that an international staging method is needed to advance research in gastritis and is preparing to test its feasibility and reproducibility both in retrospective and prospective multi-center studies.

REFERENCES

1 **Genta RM**. Inflammatory Disorders of the Stomach. In: Odze RD, Goldblum JR, Crawford DH, editors. Surgical Pathology of the GI TRact, Liver, Biliary Tract, and Pancreas. Philadelphia: Saunders, 2004: 143-176
2 **Correa P**. A human model of gastric carcinogenesis. *Cancer Res* 1988; **48**: 3554-3560
3 **Schistosomes**, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *IARC Monogr Eval Carcinog Risks Hum* 1994; **61**: 1-241
4 **Correa P**, Cuello C, Duque E, Burbano LC, Garcia FT, Bolanos O, Brown C, Haenszel W. Gastric cancer in Colombia. III. Natural history of precursor lesions. *J Natl Cancer Inst* 1976; **57**: 1027-1035
5 **Sipponen P**, Kekki M, Siurala M. Atrophic chronic gastritis and intestinal metaplasia in gastric carcinoma. Comparison with a representative population sample. *Cancer* 1983; **52**: 1062-1068
6 **You WC**, Zhang L, Gail MH, Li JY, Chang YS, Blot WJ, Zhao CL, Liu WD, Li HQ, Ma JL, Hu YR, Bravo JC, Correa P, Xu GW, Fraumeni JF Jr. Precancerous lesions in two counties of China with contrasting gastric cancer risk. *Int J Epidemiol* 1998; **27**: 945-948
7 **Correa P**. The biological model of gastric carcinogenesis. *IARC Sci Publ* 2004; (**157**): 301-310
8 **Maaroos HI**, Kekki M, Sipponen P, Salupere V, Villako K. Grade of Helicobacter pylori colonisation, chronic gastritis and relative risks of contracting high gastric ulcers: a seven-year follow-up. *Scand J Gastroenterol Suppl* 1991; **186**: 65-72
9 **Sipponen P**, Kekki M, Haapakoski J, Ihamäki T, Siurala M. Gastric cancer risk in chronic atrophic gastritis: statistical calculations of cross-sectional data. *Int J Cancer* 1985; **35**: 173-177
10 **Cassaro M**, Rugge M, Gutierrez O, Leandro G, Graham DY, Genta RM. Topographic patterns of intestinal metaplasia and gastric cancer. *Am J Gastroenterol* 2000; **95**: 1431-1438
11 **Price AB**, Misiewicz JJ. Sydney classification for gastritis. *Lancet* 1991; **337**: 174
12 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Patho*l 1996; **20**: 1161-1181
13 **Rugge M**, Correa P, Dixon MF, Fiocca R, Hattori T, Lechago J, Leandro G, Price AB, Sipponen P, Solcia E, Watanabe H, Genta RM. Gastric mucosal atrophy: interobserver consistency using new criteria for classification and grading. *Aliment Pharmacol Ther* 2002; **16**: 1249-1259
14 **Ihamäki T**, Saukkonen M, Siurala M. Long-term observation of subjects with normal mucosa and with superficial gastritis: results of 23--27 years' follow-up examinations. *Scand J Gastroenterol* 1978; **13**: 771-775
15 **Ihamäki T**, Kekki M, Sipponen P, Siurala M. The sequelae and course of chronic gastritis during a 30- to 34-year bioptic follow-up study. *Scand J Gastroenterol* 1985; **20**: 485-491
16 **Kekki M**, Ihamäki T, Varis K, Isokoski M, Lehtola J, Hovinen E, Siurala M. Age of gastric cancer patients and susceptibility to chronic gastritis in their relatives. A mathematical approach using Poisson's process and scoring of gastritis state. *Scand J Gastroenterol* 1973; **8**: 673-679
17 **Kekki M**, Villako K, Tamm A, Siurala M. Dynamics of antral and fundal gastritis in an Estonian rural population sample. *Scand J Gastroenterol* 1977; **12**: 321-324
18 **Cuello C**, Correa P, Haenszel W, Gordillo G, Brown C, Archer M, Tannenbaum S. Gastric cancer in Colombia. I. Cancer risk and suspect environmental agents. *J Natl Cancer Inst* 1976; **57**: 1015-1020
19 **Haenszel W**, Correa P, Cuello C, Guzman N, Burbano LC, Lores H, Muñoz J. Gastric cancer in Colombia. II. Case-control epidemiologic study of precursor lesions. *J Natl Cancer Inst* 1976; **57**: 1021-1026
20 **Kimura K**. Chronological transition of the fundic-pyloric border determined by stepwise biopsy of the lesser and greater curvatures of the stomach. *Gastroenterology* 1972; **63**: 584-592
21 **Hattori T**. Development of adenocarcinomas in the stomach. *Cancer* 1986; **57**: 1528-1534
22 **Correa P**. Chronic gastritis as a cancer precursor. *Scand J Gastroenterol Suppl* 1984; **104**: 131-136
23 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315
24 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52**: 6735-6740
25 **Schlemper RJ**, Riddell RH, Kato Y, Borchard F, Cooper HS, Dawsey SM, Dixon MF, Fenoglio-Preiser CM, Fléjou JF, Geboes K, Hattori T, Hirota T, Itabashi M, Iwafuchi M, Iwashita A, Kim YI, Kirchner T, Klimpfinger M, Koike M, Lauwers GY, Lewin KJ, Oberhuber G, Offner F, Price AB, Rubio CA, Shimizu M, Shimoda T, Sipponen P, Solcia E, Stolte M, Watanabe H, Yamabe H. The Vienna classification of gastrointestinal epithelial neoplasia. *Gut* 2000; **47**: 251-255
26 **Rugge M**, Correa P, Dixon MF, Hattori T, Leandro G, Lewin K, Riddell RH, Sipponen P, Watanabe H. Gastric dysplasia: the Padova international classification. *Am J Surg Pathol* 2000; **24**: 167-176
27 **Kekki M**, Ihamäki T, Varis K, Siurala M. Chronic gastritis profiles in sibs of probands calculated to carry a highly increased risk of gastric carcinoma. *Scand J Gastroenterol Suppl* 1991; **186**: 29-32
28 **Bonney GE**, Elston RC, Correa P, Haenszel W, Zavala DE, Zarama G, Collazos T, Cuello C. Genetic etiology of gastric carcinoma: I. Chronic atrophic gastritis. *Genet Epidemiol* 1986; **3**: 213-224
29 **Brenner H**, Arndt V, Stürmer T, Stegmaier C, Ziegler H, Dhom G. Individual and joint contribution of family history and Helicobacter pylori infection to the risk of gastric carcinoma. *Cancer* 2000; **88**: 274-279
30 **Ihamäki T**. Susceptibility to chronic gastritis in first degree relatives of gastric carcinoma patients. Effect of histological type of carcinoma and location of tumour. *Ann Clin Res* 1984; **16**: 183-187
31 **Ruiz B**, Garay J, Johnson W, Li D, Rugge M, Dixon MF, Fiocca R, Genta RM, Hattori T, Lechago J, Price AB, Sipponen P, Solcia E, Watanabe H, Correa P. Morphometric assessment of gastric antral atrophy: comparison with visual evaluation. *Histopathology* 2001; **39**: 235-242
32 **Ihamäki T**, Sipponen P, Varis K, Kekki M, Siurala M. Characteristics of gastric mucosa which precede occurrence of gastric malignancy: results of long-term follow-up of three family samples. *Scand J Gastroenterol Suppl* 1991; **186**: 16-23
33 **Siurala M**, Kekki M, Varis K, Isokoski M, Ihamäki T. Gastritis and gastric cancer. *Br Med J* 1972; **3**: 530-531
34 **Correa P**. Helicobacter pylori and gastric cancer: state of the art. *Cancer Epidemiol Biomarkers Prev* 1996; **5**: 477-481
35 **Miehlke S**, Hackelsberger A, Meining A, Hatz R, Lehn N, Malfertheiner P, Stolte M, Bayerdörffer E. Severe expression of corpus gastritis is characteristic in gastric cancer patients infected with Helicobacter pylori. *Br J Cancer* 1998; **78**: 263-266
36 **Walker IR**, Strickland RG, Ungar B, Mackay IR. Simple atrophic gastritis and gastric carcinoma. *Gut* 1971; **12**: 906-911
37 **Sipponen P**, Stolte M. Clinical impact of routine biopsies of

the gastric antrum and body. *Endoscopy* 1997; **29**: 671-678
38 **Jass JR**, Filipe MI. Sulphomucins and precancerous lesions of the human stomach. *Histopathology* 1980; **4**: 271-279
39 **Jass JR**, Filipe MI. The mucin profiles of normal gastric mucosa, intestinal metaplasia and its variants and gastric carcinoma. *Histochem J* 1981; **13**: 931-939
40 **Tosi P**, Filipe MI, Luzi P, Miracco C, Santopietro R, Lio R, Sforza V, Barbini P. Gastric intestinal metaplasia type III cases are classified as low-grade dysplasia on the basis of morphometry. *J Pathol* 1993; **169**: 73-78
41 **Filipe MI**, Potet F, Bogomoletz WV, Dawson PA, Fabiani B, Chauveinc P, Fenzy A, Gazzard B, Goldfain D, Zeegen R. Incomplete sulphomucin-secreting intestinal metaplasia for gastric cancer. Preliminary data from a prospective study from three centres. *Gut* 1985; **26**: 1319-1326
42 **Filipe MI**, Muñoz N, Matko I, Kato I, Pompe-Kirn V, Jutersek A, Teuchmann S, Benz M, Prijon T. Intestinal metaplasia types and the risk of gastric cancer: a cohort study in Slovenia. *Int J Cancer* 1994; **57**: 324-329
43 **Leung WK**, Sung JJ. Review article: intestinal metaplasia and gastric carcinogenesis. *Aliment Pharmacol Ther* 2002; **16**: 1209-1216
44 **Riddell RH**, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC. Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum Pathol* 1983; **14**: 931-968
45 **Haggitt RC**. Barrett's esophagus, dysplasia, and adenocarcinoma. *Hum Pathol* 1994; **25**: 982-993
46 **Reid BJ**, Haggitt RC, Rubin CE, Roth G, Surawicz CM, Van Belle G, Lewin K, Weinstein WM, Antonioli DA, Goldman H. Observer variation in the diagnosis of dysplasia in Barrett's esophagus. *Hum Pathol* 1988; **19**: 166-178
47 **Ming SC**, Bajtai A, Correa P, Elster K, Jarvi OH, Munoz N, Nagayo T, Stemmerman GN. Gastric dysplasia. Significance and pathologic criteria. *Cancer* 1984; **54**: 1794-1801
48 **Genta RM**. Gastric Dysplasia in the East and West. Current Gastroenterology Reports 2; In press
49 **Schlemper RJ**, Itabashi M, Kato Y, Lewin KJ, Riddell RH, Shimoda T, Sipponen P, Stolte M, Watanabe H, Takahashi H, Fujita R. Differences in diagnostic criteria for gastric carcinoma between Japanese and western pathologists. *Lancet* 1997; **349**: 1725-1729
50 **Fenoglio-Preiser CM**, Carneiro F, Correa P, Guilford P, Lambert R, Megraud F et al. Gastric carcinoma. In: Hamilton SR, Aaltonen LA, editors. World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of the Digestive System. Lyon: IARC Press, 2000: 37-68
51 **Schlemper RJ**, Kato Y, Stolte M. Diagnostic criteria for gastrointestinal carcinomas in Japan and Western countries: proposal for a new classification system of gastrointestinal epithelial neoplasia. *J Gastroenterol Hepatol* 2000; **15** Suppl: G49-G57
52 **Japanese Research Society for Gastric Cancer**. Japanese Classification of Gastric Carcinoma. 1st ed. English Edition. Tokyo: Kanehara & Co., Ltd., 1995
53 **El-Zimaity HM**, Graham DY. Evaluation of gastric mucosal biopsy site and number for identification of Helicobacter pylori or intestinal metaplasia: role of the Sydney System. *Hum Pathol* 1999; **30**: 72-77
54 **Rugge M**, Farinati F, Baffa R, Sonego F, Di Mario F, Leandro G, Valiante F. Gastric epithelial dysplasia in the natural history of gastric cancer: a multicenter prospective follow-up study. Interdisciplinary Group on Gastric Epithelial Dysplasia. *Gastroenterology* 1994; **107**: 1288-1296
55 **Rugge M**, Cassaro M, Di Mario F, Leo G, Leandro G, Russo VM, Pennelli G, Farinati F. The long term outcome of gastric non-invasive neoplasia. *Gut* 2003; **52**: 1111-1116
56 **Genta RM**, Graham DY. Primary gastric MALT lymphoma: trivial condition or serious disease? *Helicobacter* 1997; **2** Suppl 1: S56-S60
57 **Korstanje A**, den Hartog G, Biemond I, Lamers CB. The serological gastric biopsy: a non-endoscopical diagnostic approach in management of the dyspeptic patient: significance for primary care based on a survey of the literature. *Scand J Gastroenterol Suppl* 2002; **236**: 22-26
58 **Di Mario F**, Kusstatscher S, Ferrana M, Dal Bo' N, Plebani M, Rugge M. Helicobacter pylori eradication and serum pepsinogens. *Gut* 1996; **38**: 793
59 **Di Mario F**, Moussa AM, Caruana P, Merli R, Cavallaro LG, Cavestro GM, Dal Bò N, Iori V, Pilotto A, Leandro G, Franzè A, Rugge M. 'Serological biopsy' in first-degree relatives of patients with gastric cancer affected by Helicobacter pylori infection. *Scand J Gastroenterol* 2003; **38**: 1223-1227
60 **Sipponen P**, Härkönen M, Alanko A, Suovaniemi O. Diagnosis of atrophic gastritis from a serum sample. *Clin Lab* 2002; **48**: 505-515
61 **Mårdh E**, Mårdh S, Mårdh B, Borch K. Diagnosis of gastritis by means of a combination of serological analyses. *Clin Chim Acta* 2002; **320**: 17-27
62 **Plebani M**, Di Mario F, Vianello F, Farini R, Piccoli A, Lazzaretto L, Perobelli L, Naccarato R, Burlina A. Actual role of pepsinogen group I in the study of upper gastrointestinal diseases. *Clin Biochem* 1983; **16**: 310-312
63 **Plebani M**, Di Mario F, Stanghellini V, Delle Fave G. Serological tests to monitor treatment of Helicobacter pylori. *Lancet* 1992; **340**: 51-52
64 **Farinati F**, Di Mario F, Plebani M, Cielo R, Fanton MC, Valiante F, Masiero M, De Boni M, Della Libera G, Burlina A. Pepsinogen A/pepsinogen C or pepsinogen A multiplied by gastrin in the diagnosis of gastric cancer? *Ital J Gastroenterol* 1991; **23**: 194-196
65 **Varis K**, Kekki M, Härkönen M, Sipponen P, Samloff IM. Serum pepsinogen I and serum gastrin in the screening of atrophic pangastritis with high risk of gastric cancer. *Scand J Gastroenterol Suppl* 1991; **186**: 117-123
66 **Varis K**, Sipponen P, Laxén F, Samloff IM, Huttunen JK, Taylor PR, Heinonen OP, Albanes D, Sande N, Virtamo J, Härkönen M. Implications of serum pepsinogen I in early endoscopic diagnosis of gastric cancer and dysplasia. Helsinki Gastritis Study Group. *Scand J Gastroenterol* 2000; **35**: 950-956
67 **Germaná B**, Di Mario F, Cavallaro LG, Moussa AM, Lecis P, Liatoupolou S, Comparato G, Carloni C, Bertiato G, Battiestel M, Papa N, Aragona G, Cavestro GM, Iori V, Merli R, Bertolini S, Caruana P, Franze A. Clinical usefulness of serum pepsinogens I and II, gastrin-17 and anti-Helicobacterpylori antibodies in the management of dyspeptic patients in primary care. *Dig Liver Dis* 2005; **37**: 501-508
68 ISI Web of Science-Citation Index. Science Citation Index Expanded (SCI-Expanded)-1945-present, 2004
69 **Rugge M**, Genta RM. Staging and grading of chronic gastritis. *Hum Pathol* 2005; **36**: 228-233
70 **Desmet VJ**, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513-1520
71 **Ishak K**, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696-699

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GASTRIC CANCER

Resveratrol engages selective apoptotic signals in gastric adenocarcinoma cells

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Abstract

AIM: To investigate the intracellular apoptotic signals engaged by resveratrol in three gastric adenocarcinoma cancer cell lines, two of which (AGS and SNU-1) express p53 and one (KATO-Ⅲ) with deleted p53.

METHODS: Nuclear fragmentation was used to quantitate apoptotic cells; caspase activity was determined by photometric detection of cleaved substrates; formation of oxidized cytochrome C was used to measure cytochrome C activity, and Western blot analysis was used to determine protein expression.

RESULTS: Gastric cancer cells, irrespective of their p53 status, responded to resveratrol with fragmentation of DNA and cleavage of nuclear lamins A and B and PARP. Resveratrol, however, has no effect on mitochondria-associated apoptotic proteins Bcl-2, Bcl-xl, Bax, Bid or Smac/Diablo, and did not promote subcellular redistribution of cytochrome C, indicating that resveratrol-induced apoptosis of gastric carcinoma cells does not require breakdown of mitochondrial membrane integrity. Resveratrol up-regulated p53 protein in SNU-1 and AGS cells but there was a difference in response of intracellular apoptotic signals between these cell lines. SNU-1 cells responded to resveratrol treatment with down-regulation of survivin, whereas in AGS and KATO-Ⅲ cells resveratrol stimulated caspase 3 and cytochrome C oxidase activities.

CONCLUSION: These findings indicate that even within a specific cancer the intracellular apoptotic signals engaged by resveratrol are cell type dependent and suggest that such differences may be related to differentiation or lack of differentiation of these cells.

INTRODUCTION

Gastric cancer is a major cause of mortality both in developed and underdeveloped countries because currently available chemotherapeutic regimens are not very effective, resulting in high recurrence rates and poor survival. There is strong evidence that the predominant etiological factors contributing to development of gastric cancer are infections with *H pylori* during early years of life and/or exposure to chemical carcinogens such as those in cigarettes and cured meat[1]. Identification and eradication of *H pylori* in the world population would be an economically prohibitive undertaking because more than 50% of population over the age of 50 are infected with the bacterium, and eradiation would not benefit those with pre-malignant gastric mucosal alterations. However, given the epigenetic origin and prolonged onset of gastric cancer development, the concept of cancer chemoprevention presents an attractive hypothesis to reduce the risk of gastric cancer.

Since apoptosis-inducing compounds control cancer cell proliferation, it is feasible that cancer development may be arrested through molecular intervention with compounds that retard cellular proliferation and induce apoptosis. Trans-resveratrol, a polyphenol found in grapes, wine and peanuts, presents itself as a dietary chemopreventive because numerous studies have demonstrated its ability to suppress proliferation and induce apoptosis in a variety of transformed cells[2]. We have shown that gastric adenocarcinoma cells respond to resveratrol with inhibition of DNA synthesis, cell cycle arrest, suppressed proliferation, and induction of apoptosis[3,4], and there is evidence that resveratrol inhibits the growth of transplanted gastric tumor[5].

There are two major pathways for induction of apoptosis: (1) the extrinsic pathway activated when

extracellular ligands interact with receptors of the TNF family (TNF, FAS, and TRAIL) and (2) the intrinsic pathway, induced by destabilization of mitochondria. Resveratrol up-regulates Fas and Fas-L in gastric adenocarcinoma cells that express p53, whereas only Fas-L becomes up-regulated in cells whose p53 is deleted[6], suggesting that apoptotic signals engaged by resveratrol within individual gastric carcinoma cell lines may be dependent on p53 status of the cell. Here we explore the action of resveratrol on intracellular apoptotic signals in three different gastric adenocarcinoma cell lines.

MATERIALS AND METHODS

Materials

Resveratrol is a kind gift from Pharmascience, Montreal, Quebec, Canada. The following caspase substrates were purchased from Calbiochem, EMD Biosciences, Inc., San Diego, CA: caspase 1 substrate (Ac-WEHD-pNA), caspase 3 substrate (Ac-DEVD-pNA), caspase 6 substrate (Ac-VEID-pNA), caspase 8 substrate (Ac-IETD-pNA), and caspase 9 substrate (Ac-LEHD-pNA). Cytochrome C Oxidase Assay Kit (CYTOC-OX1) was purchased from Sigma-Aldrich, St. Louis, MO. Antibodies to cytochrome C and lamin B were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; antibodies to PARP, Bcl-xl, Bax, Bid, and cleaved Lamin A were from Cell Signaling Biotechnology, Beverly, MA; antibody to survivin was from Novus Biologicals, Inc., Littleton, CO; p53 antibody was from PharMingen, Inc/BD Biosciences, San Diego, CA; antibody to β-actin was from Sigma-Aldrich, St. Louis, MO, and antibodies to Bcl-2 and Smac/DIABLO were from Calbiochem/EMD Biosciences, Inc., La Jolla, CA. Chemiluminescence detection system (ECL Western Blotting System) was from Amersham/Pharmacia Biotech, Piscataway, NJ.

Cell culture and cell treatment

Human gastric adenocarcinoma cell lines AGS (ATCC: CLR-1739), SNU-1 (ATCC: CRL-5971) and KATO-III (ATCC: HTB 103) were routinely cultured in RPMI-1640 media supplemented with 100 mL/L fetal bovine serum (FBS), 10 U/mL of streptomycin and 0.25 mg/L of amphotericin B at 37℃ in humidified air with 50 mL/L CO_2. Cells were allowed to equilibrate in fresh media for 2-3 h prior to addition of resveratrol which was dissolved in 950 mL/L ethanol. The concentration of ethanol was always maintained at 0.1% in both treated and untreated cells. A stock solution of 100 mmol/L resveratrol was prepared weekly and stored in the dark at -20℃.

Determination of apoptosis

Percent of apoptotic cells was determined using Cell Death Detection ELISA^{PLUS} kit from Roche Applied Science, Indianapolis, IN. Cells (1×10^4 cells/200 μL) were incubated for designated times with or without 100 μmol/L resveratrol, and percent of apoptotic cells was calculated based on 100% apoptosis obtained after 48 h of cell exposure to 50 μmol/L camptothecin.

Caspase activity measurements

Cells were plated in fresh media at 1×10^6 cells/5 mL in 6 well plates, allowed to equilibrate for 3 h, and treated with 100 μmol/L resveratrol for 24 or 48 h. Vehicle was added to untreated controls that were also cultured for 24 or 48 h. At the end of the specified incubation time, cells were harvested, washed once with 5 mL of PBS and lysed for 30 min at 4℃ in lysis buffer A (10 mmol/L Tris pH 7.4, 150 mmol/LNaCl, 1 mmol/L EDTA, 1 mmol/LEGTA, 1% Triton X-100, 0.2 mmol/L sodium vanadate, 2 mmol/L phenylmethyl-sulfonyl fluoride, 0.5% NP-40, and 20 mmol/L NaF). The caspase assay was performed in 96 well plates in a total volume of 100 μL as follows: to 32 μL of assay buffer (312.5 mmol/L HEPES, 31.25% glucose, 0.3125% CHAPS) were added 2 μL of DMSO, 10 μL of 100 mmol/L DTT, 30 μg of cell lysate protein, and the volume adjusted with deionized water to 98 μL. Following addition of 2 μL of 10 mmol/L fluorogenic peptide substrate, specific for each caspase, the reaction was incubated at 25℃ for 4 h after which time absorbance measured at 405 nm on a microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Activity of each caspase activity was calculated from a standard curve of p-nitroaniline (pNA) absorbance at 405 nm and values are expressed as pmoles of pNA generated per mg lysate protein. Protein content of cell lysates was determined by the method of Lowry[7].

Western blot analysis

Cellular levels of cytochrome C, p53, survivin, Bax, Bcl-2, Bcl-xl, Bid, Smac/DIABLO, cleaved lamin A, lamin B, PARP, and β-actin (used as control for equal protein loading) were determined in untreated and resveratrol treated cell fractions by Western blot detection. To test the action of resveratrol, cells (0.2×10^9 cells/L of media) were treated with 100 μmol/L resveratrol for 24 or 48 h, after which time they were harvested, washed with PBS, suspended in 0.5 mL of lysis buffer B (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 0.2 mmol/L sodium vanadate, 2 mmol/L phenylmethyl-sulfonyl fluoride, 0.5% NP-40, 20 mmol/L NaF, and 2 mg/L leupeptin), and rapidly frozen in liquid nitrogen. Cells were lysed by a freeze-thaw cycle in liquid nitrogen followed by 30 min at 4℃ and cell cytosol was prepared from cell lysates by centrifugation at 10 000 × *g* for 20 min. Mitochondria were prepared from whole cells by a subcellular fractionation protocol[8] and protein content of each fraction determined by the Lowry method[7]. Equal amounts of protein were separated by SDS-PAGE on either 15% resolving Tris-HCl gels for detection of cytochrome C, survivin, Bcl-2, Bcl-xl, Bax, Bid, and Smac/DIABLO or 12% gels for detection of p53, PARP, cleaved lamin A, lamin B, β-actin, followed by transfer to PVDF membranes, and antigen detected by chemiluminescence. Depicted results are representative of at least two individual experiments.

Measurement of cytochrome C oxidase activity

Cytochrome C oxidase activity was measured in cell

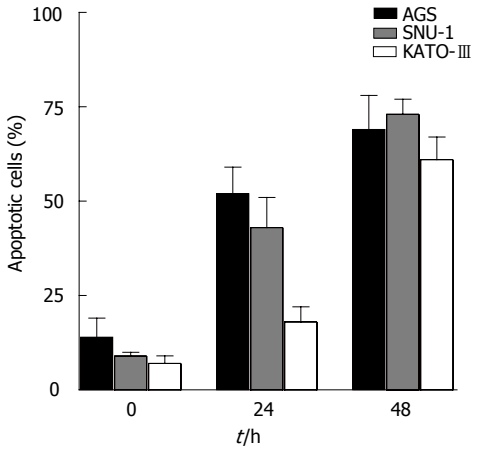

Figure 1 Effect of resveratrol on apoptosis of gastric adenocarcinoma cells.

lysates from untreated and resveratrol treated cells using a commercially available kit from Sigma-Aldrich. Activity of cytochrome C oxidase was determined by measuring the conversion of reduced cytochrome C, which absorbs light at A_{50}, to its oxidized form that does not absorb light at this wavelength, and values are expressed as ΔA_{550} generated by 35 μg of lysate protein.

Statistical analysis

Numerical data were analyzed for statistical significance by Student's *t* test, and Results are expressed as the mean ± SE. Statistical significance is denoted as $P < 0.01$ and $P < 0.05$.

RESULTS

Effect of resveratrol on apoptosis of SNU-1 cells

Gastric adenocarcinoma SNU-1 cells (expressing p53) and Kato-Ⅲ cells (p53 deleted) respond to resveratrol treatment with decreased proliferation, concentration dependent inhibition of DNA synthesis and cell cycle arrest[3,4]. To determine whether p53 status of the cell regulates the action of resveratrol on apoptosis, we measured the extent of apoptosis in three gastric adenocarcinoma cells lines: AGS (expressing wild type p53), SNU-1 (expressing p53) and KATO-Ⅲ. Results show that cell treatment with 100 μmol/L resveratrol induces a time dependent apoptosis in all three cell lines (Figure 1). Although a small percentage of apoptotic cells was present in the absence of resveratrol, treatment with 100 μmol/L resveratrol for 24 h resulted in significantly increased accumulation of apoptotic cells, and treatment for 48 h further increasing the percentage of apoptotic cells. After 48 h of treatment with 100 μmol/L resveratrol nearly 70% of AGS cells, 75% of SNU-1 cells and 62% of Kato-Ⅲ cells became apoptotic.

Action of resveratrol on nuclear targets of apoptosis

Apoptosis is characterized by loss of nuclear integrity and degradation of DNA and we previously demonstrated that exposure of gastric adenocarcinoma cells to resveratrol results in DNA fragmentation[3,4]. Here we determined the action of resveratrol on nuclear proteins PARP, lamin

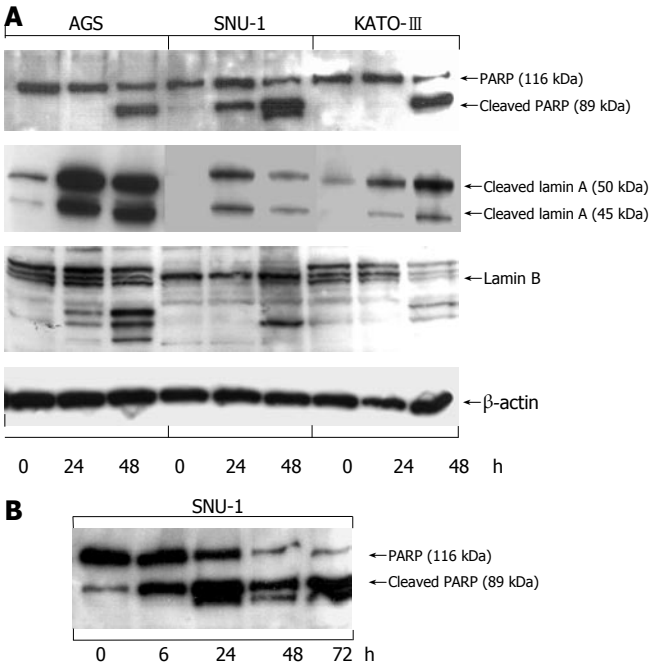

Figure 2 Effect of resveratrol on lamin A, lamin B (**A**) and PARP (**B**).

A and lamin B (Figure 2A), and show that resveratrol promotes their cleavage in all three cell lines. Increased levels of lamin A cleavage products were seen in all cells within 24 h of resveratrol treatment, whereas lamin B cleavage products were seen in AGS cells after 24 h, while SNU-1 and Kato-Ⅲ cells required 48 h of exposure to 100 μmol/L resveratrol to induce lamin B cleavage. Resveratrol treatment caused cleavage of the 116 kDa PARP to an 89 kDa fragment in all three cell lines although there was a time difference among the individual cell lines. In SNU-1 cells the 89 kDa breakdown product of PARP was detected after 24 h while in AGS and KATO-Ⅲ cells PARP breakdown product was seen after 48 h. Closer analysis of PARP in SNU-1 cells revealed presence of the 89 kDa fragment within 6 h after cell exposure to resveratrol with further breakdown of the 89 kDa band to a smaller fragment after prolonged treatment and nearly total loss of the 116 kDa PARP protein after 72 h of cell exposure to 100 μmol/L (Figure 2B).

Action of resveratrol on caspases

Because nuclear proteins become cleaved during resveratrol-induced apoptosis, we investigated whether this action of resveratrol results from activation of caspases. Activities of caspases 1, 3, 6, 8, and 9 were measured in untreated cells and in cells treated for 24 or 48 h with 100 μmol/L resveratrol. Results show that 100 μmol/L resveratrol had no effect on caspases 1, 8, and 9, but significantly stimulated caspase 3 activity in AGS and KATO-Ⅲ cells and caused some activation of caspase 6 in AGS cells after 24 h. Caspase 3 activity in AGS cells was increased after 24 h of treatment and remained elevated after 48 h, whereas in KATO-Ⅲ cells caspase 3 activity responded to resveratrol only after 48 h. Resveratrol, however, had no effect on caspase 3 activity in SNU-1 cells (Figure 3).

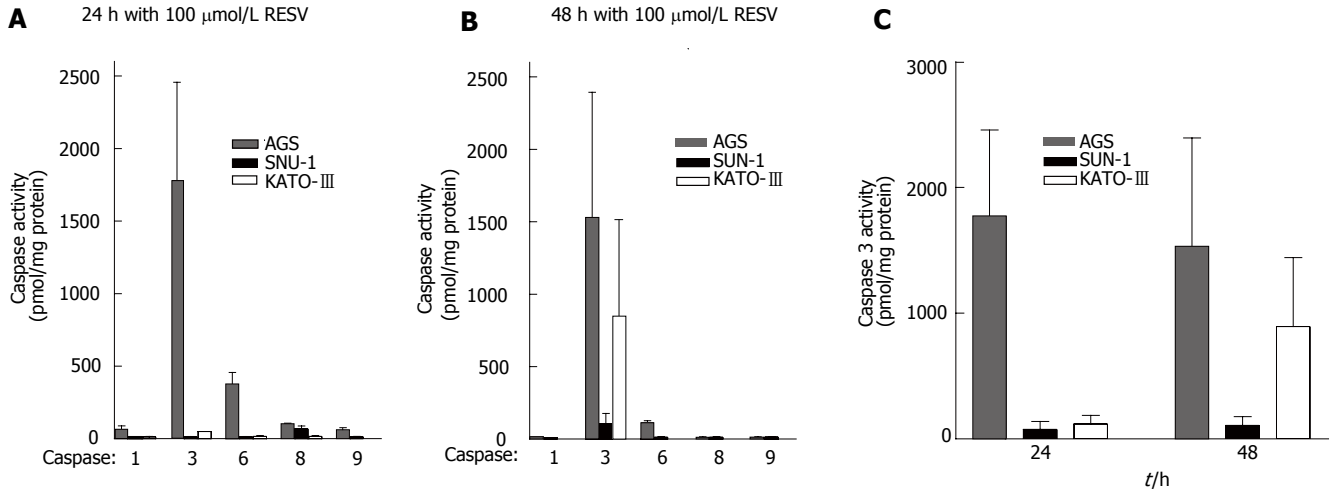

Figure 3 Effect of resveratrol on caspase acitivity.

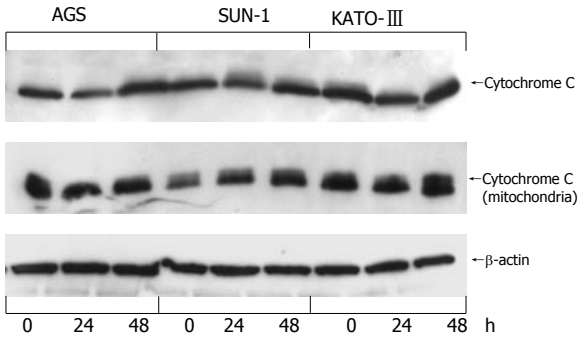

Figure 4 Effect of resveratrol on subcellular distribution of cytochrome C.

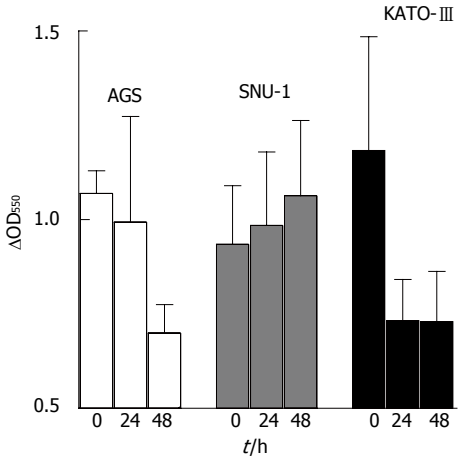

Figure 5 Action of resveratrol on cytochrome C oxidase activity.

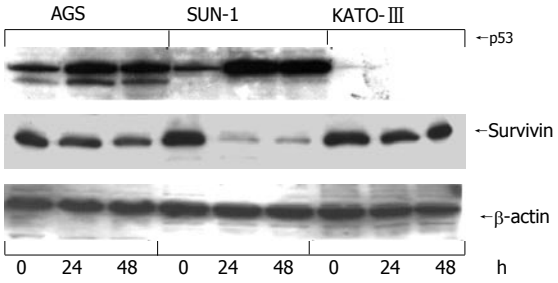

Figure 6 Effect of resveratrol on p53 and survivin.

Action of resveratrol on subcellular distribution of cytochrome C

Numerous cytotoxic reagents, radiation, and growth factor withdrawal induce apoptosis by promoting release of mitochondrial cytochrome C into cell cytosol. To determine whether resveratrol targets mitochondrial permeability in gastric adenocarcinoma cells we measured distribution of cytochrome C protein between cell cytosol and mitochondria after 24 and 48 h of cell treatment with 100 μmol/L resveratrol. Results indicate that cytochrome C was found to be present in both mitochondria and cytosol of each cell line prior to treatment with resveratrol, and resveratrol treatment had no further effect on redistribution of cytochrome C protein between these two compartments (Figure 4).

Action of resveratrol on cytochrome C oxidase activity

In addition to its role in apoptosis, cytochrome C functions in the respiratory chain as carrier of electrons from flavoproteins to cytochrome oxidase. Since resveratrol is a known antioxidant and inhibits mitochondrial respiratory chain[9], we inquired whether cytochrome C contributes to the antioxidant potential of resveratrol by measuring cytochrome C oxidase activity in untreated as well as resveratrol treated cells. Results show that resveratrol had a significant stimulatory effect on cytochrome C oxidase activity in KATO-Ⅲ cells after 24 h of cell treatment, and in AGS cells after 48 of treatment, but had no effect on cytochrome C oxidase activity in SNU-1 cells (Figure 5).

Action of resveratrol on p53 and survivin levels

Cellular levels of survivin, an inhibitor of apoptosis, have been shown to correlate inversely with expression of the p53 tumor suppressor[10-12], and we determined the action of resveratrol on survivin and p53 protein levels in AGS, SNU-1 and Kato-Ⅲ cells. Results presented in Figure 6 indicate that resveratrol up-regulates p53 protein in AGS and SNU-1 cells but has no effect on the p53 status in Kato-Ⅲ cells. Within 24 h after exposure to 100 μmol/L

resveratrol survivin levels in SNU-1 cells became down-regulated. Although AGS cells express p53 and its p53 is up-regulated by resveratrol, levels of survivin in AGS cells were not altered by resveratrol, and resveratrol had no effect on survivin in KATO-Ⅲ cells.

Action of resveratrol on Bcl-2 proteins

To evaluate the contribution of pro- and anti-apoptotic Bcl-2 proteins in the response of gastric carcinoma cells to resveratrol we measured protein levels of anti-apoptotic Bcl-2 and Bcl-xl and pro-apoptotic Bax, as well as Bid and Smac/DIABLO. Cells were treated with 100 μmol/L resveratrol for 24 or 48 h, sub-cellular fractions prepared as described in Methods, and protein levels determined by Western blotting. Isolated mitochondria were used to determine Bcl-2 and Bcl-xl levels, whereas Bax, Bid and Smac/DIABLO were measured in cell cytosol. Results presented in Figure 7 show that cell treatment with 100 μmol/L resveratrol for up to 48 h had no effect on Bax, Bcl-2, Bcl-xl, and Smac/DIABLO, and did not promote cleavage of cytosolic 22 kDa Bid to the active 15-17 kDa form.

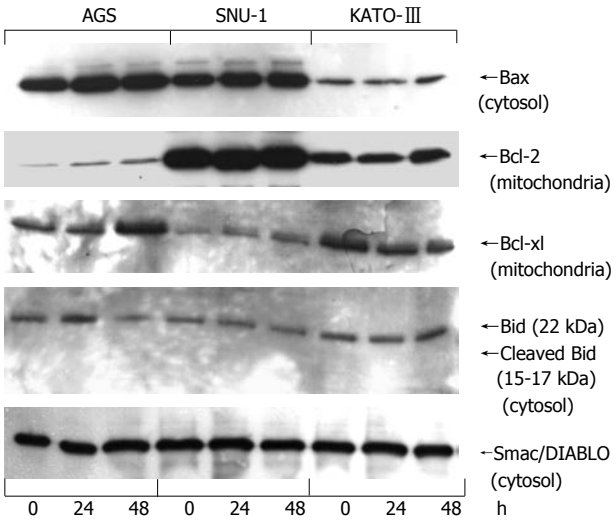

Figure 7 Effect of resveratrol on Bax, Bcl-2, Bcl-xl, Bid and Smac/DIABLO.

DISCUSSION

A common feature of malignant cells is their ability to proliferate without restraint and, therefore, apoptosis is considered the predominant pathway for elimination of malignant cells with the signals engaged by an apoptotic agent determining its efficacy as a chemopreventive or as an adjuvant to chemotherapy. Previous work from this laboratory has shown that resveratrol-induced engagement of Fas receptor is dependent on the p53 status of the cells. The aim of the present investigation was to determine whether intracellular apoptotic signals engaged by resveratrol are also regulated to some extent by p53. To enhance the signaling response to resveratrol all experiments were performed using 100 μmol/L because this concentration has been repeatedly shown to promote significant apoptotic response in human gastric adenocarcinoma cells[3-5] and in other malignant cells[13-15].

One of the differences among the three gastric carcinoma cell lines used in this study is their p53 status: Kato-Ⅲ cells do not express p53, whereas p53 is present in both AGS and SNU-1 cells. Resveratrol induced a time-dependent apoptotic response in all three cell lines irrespective of their p53 status, and in each cell line resveratrol-induced apoptosis was associated with cleavage of PARP, lamin A and lamin B. Other studies investigating the involvement of p53 in cellular response to resveratrol have also shown that resveratrol induces apoptosis and up-regulates p53 in cells that express this tumor suppressor[16,17], and that it induces apoptosis in p53 deficient cells[18,19]. Tumor suppressor p53 exerts control over proliferation and apoptosis by initiating transcriptional activation of specific genes, among them the gene for survivin[10-12], an inhibitor of apoptosis found to be expressed in all types of malignancies but not in normal, differentiated cells. Patients with gastric cancer express increased abundance of survivin[20] and survivin expression correlates with p53 accumulation[18], whereas suppression of survivin inhibits growth of gastric cancer cells and decreases tumorigenesis[21]. In our study resveratol increased p53 levels in both AGS and SNU-1 cells, but only p53-expressing SNU-1 cells responded to resveratrol treatment with loss of survivin suggesting that p53 in AGS cells may have mutations, a common finding in tumor cells.

Since resveratrol is a small, lipophylic molecule it can intercalate within the mitochondrial membrane and directly induce apoptosis through destabilization of mitochondrial membrane. To address this question we determined action of resveratrol action on Bcl-2 family of apoptotic regulators. These proteins control mitochondrial permeability and promote release of mitochondrial cytochrome C. Our findings revealed that resveratrol had no effect on either anti-apoptotic Bax or pro-apoptotic Bcl-2 and Bcl-xl, did not promote cleavage of Bid and had no effect on subcellular redistribution of cytochrome C. On the basis of these results we conclude that resveratrol-induced apoptosis of gastric cancer cells in culture is not an outcome of mitochondrial integrity breakdown. The action of resveratrol on Bcl-2 proteins has been investigated in a number of cells with results supporting a cell type-dependent response. Human leukemia U937 cells readily succumbed to apoptosis after treatment with 100 μmol/L resveratrol, whereas apoptosis was significantly inhibited when these cells were modified to over-express Bcl-2[22]. In non-Hodgkin's lymphoma and multiple myeloma cell lines Bcl-xl expression was down-regulated by resveratrol[23], but resveratrol had no effect on Bcl-xl, Bcl-2 nor Bax in other malignant cells[24], and Bax levels remained unchanged in colorectal carcinoma cells after 24 h of treatment with 40 μmol/L resveratrol[25]. Since resveratrol caused a decrease in the ratio of Bcl-2/Bax in transplanted gastric tumors and we can only conclude that cells in culture respond differently to resveratrol from primary gastric cancer cells transplanted into nude mice.

Destabilization of mitochondria usually results in release of mitochondrial cytochrome C into cells cytosol, but we found no evidence of involvement of

mitochondria destabilizing Bcl-2 proteins in resveratrol-induced apoptosis of gastric carcinoma cells. However, we did observe presence of cytochrome C in cytosol of untreated as well as resveratrol treated cells. Cytosolic cytochrome C has also been identified within secretory granules of normal rat pancreas and anterior pituitary[26], and because secretory granules are present in gastric carcinoma cells[27,28], we addressed the possible role of cytosolic cytochrome C by determining cytochrome C oxidase activity. Our data revealed that resveratrol stimulates cytochrome C oxidase activity in AGS and KATO-Ⅲ cells, an action that would contribute to the antioxidant potential of these cells. Since transformed cells generate low levels of reactive oxygen that activate gene transcription and stimulate their proliferation[29,30], antioxidants suppress generation of endogenous reactive oxygen and are considered antiproliferative. We previously demonstrated that resveratrol is antiproliferative and behaves as an antioxidant in gastric adenocarcinoma cells[3,4]. Our current finding that resveratrol stimulates cytochrome C oxidase is a feasible mechanism for its antioxidant and antiproliferative action toward these. Inhibition of proliferation and induction of apoptosis are known to be closely related events, and when activation of cytochrome C oxidase is coupled with increased casapse 3 activity, as was seen in AGS and KATO-Ⅲ cells, these events might provide sufficient stimuli to inhibit cellular proliferation and induce apoptosis. Although resveratrol had no effect on either cytochrome C oxidase or caspase 3 in SNU-1 cells, apoptosis in SNU-1 cells probably results from down-regulation of survivin and removal of its protective mechanisms, as was demonstrated for human bladder cells[31].

In summary, our results reveal that individual gastric carcinoma cell lines respond to resveratrol with engagement of individual apoptotic signals. In p53 expressing SNU-1 cells resveratrol up-regulated p53 and down-regulated survivin, whereas in KATO-Ⅲ cells and in AGS cells resveratrol stimulated caspase 3 and cytochrome C oxidase activities, enabling suppression of proliferation while stimulating breakdown of nuclear proteins. These findings indicate that even within a specific disease resveratrol can engage alternate apoptotic targets thus providing further evidence that resveratrol can be considered a versatile chemopreventive agent.

REFERENCES

1 **Blankfield RP**. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2002; **346**: 65-67
2 **Bhat KP**, Pezzuto JM. Cancer chemopreventive activity of resveratrol. *Ann N Y Acad Sci* 2002; **957**: 210-229
3 **Atten MJ**, Attar BM, Milson T, Holian O. Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism. *Biochem Pharmacol* 2001; **62**: 1423-1432
4 **Holian O**, Wahid S, Atten MJ, Attar BM. Inhibition of gastric cancer cell proliferation by resveratrol: role of nitric oxide. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G809-G816
5 **Zhou HB**, Chen JJ, Wang WX, Cai JT, Du Q. Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice. *World J Gastroenterol* 2005; **11**: 280-284
6 **Atten MJ**, Godoy-Romero E, Attar BM, Milson T, Zopel M, Holian O. Resveratrol regulates cellular PKC alpha and delta to inhibit growth and induce apoptosis in gastric cancer cells. *Invest New Drugs* 2005; **23**: 111-119
7 **LOWRY OH**, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275
8 **Rice EJ**, Lindsay G. Subcellular fractionation of mitochondria. Subcellular Fractionation: A practical approach. Oxford: Oxford Univ Press, 1997: 107-142
9 **Zini R**, Morin C, Bertelli A, Bertelli AA, Tillement JP. Effects of resveratrol on the rat brain respiratory chain. *Drugs Exp Clin Res* 1999; **25**: 87-97
10 **Lu CD**, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. *Cancer Res* 1998; **58**: 1808-1812
11 **Hoffman WH**, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* 2002; **277**: 3247-3257
12 **Grossman D**, Kim PJ, Blanc-Brude OP, Brash DE, Tognin S, Marchisio PC, Altieri DC. Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53. *J Clin Invest* 2001; **108**: 991-999
13 **Jang M**, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 1997; **275**: 218-220
14 **Godichaud S**, Krisa S, Couronné B, Dubuisson L, Mérillon JM, Desmoulière A, Rosenbaum J. Deactivation of cultured human liver myofibroblasts by trans-resveratrol, a grapevine-derived polyphenol. *Hepatology* 2000; **31**: 922-931
15 **Niles RM**, McFarland M, Weimer MB, Redkar A, Fu YM, Meadows GG. Resveratrol is a potent inducer of apoptosis in human melanoma cells. *Cancer Lett* 2003; **190**: 157-163
16 **Huang C**, Ma WY, Goranson A, Dong Z. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis* 1999; **20**: 237-242
17 **Laux MT**, Aregullin M, Berry JP, Flanders JA, Rodriguez E. Identification of a p53-dependent pathway in the induction of apoptosis of human breast cancer cells by the natural product, resveratrol. *J Altern Complement Med* 2004; **10**: 235-239
18 **Mahyar-Roemer M**, Katsen A, Mestres P, Roemer K. Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. *Int J Cancer* 2001; **94**: 615-622
19 **Bernhard D**, Tinhofer I, Tonko M, Hübl H, Ausserlechner MJ, Greil R, Kofler R, Csordas A. Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acute leukemia cells. *Cell Death Differ* 2000; **7**: 834-842
20 **Yu J**, Leung WK, Ebert MP, Ng EK, Go MY, Wang HB, Chung SC, Malfertheiner P, Sung JJ. Increased expression of survivin in gastric cancer patients and in first degree relatives. *Br J Cancer* 2002; **87**: 91-97
21 **Tu SP**, Jiang XH, Lin MC, Cui JT, Yang Y, Lum CT, Zou B, Zhu YB, Jiang SH, Wong WM, Chan AO, Yuen MF, Lam SK, Kung HF, Wong BC. Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer. *Cancer Res* 2003; **63**: 7724-7732
22 **Park JW**, Choi YJ, Suh SI, Baek WK, Suh MH, Jin IN, Min DS, Woo JH, Chang JS, Passaniti A, Lee YH, Kwon TK. Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity. *Carcinogenesis* 2001; **22**: 1633-1639
23 **Jazirehi AR**, Bonavida B. Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis. *Mol Cancer Ther* 2004; **3**: 71-84
24 **Fulda S**, Debatin KM. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by the chemopreventive agent resveratrol. *Cancer Res* 2004; **64**: 337-346

25 **Mahyar-Roemer M**, Köhler H, Roemer K. Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells. *BMC Cancer* 2002; **2**: 27
26 **Soltys BJ**, Andrews DW, Jemmerson R, Gupta RS. Cytochrome-C localizes in secretory granules in pancreas and anterior pituitary. *Cell Biol Int* 2001; **25**: 331-338
27 **Waldum HL**, Aase S, Kvetnoi I, Brenna E, Sandvik AK, Syversen U, Johnsen G, Vatten L, Polak JM. Neuroendocrine differentiation in human gastric carcinoma. *Cancer* 1998; **83**: 435-444
28 **Koyama S**, Ebihara T, Osuga T. Histologic and immunohistochemical studies of alpha-fetoprotein (AFP)-producing gastric carcinoma. *Gastroenterol Jpn* 1987; **22**: 419-427
29 **Irani K**, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T, Goldschmidt-Clermont PJ. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science* 1997; **275**: 1649-1652
30 **Irani K**, Goldschmidt-Clermont PJ. Ras, superoxide and signal transduction. *Biochem Pharmacol* 1998; **55**: 1339-1346
31 **Tyagi AK**, Agarwal C, Singh RP, Shroyer KR, Glode LM, Agarwal R. Silibinin down-regulates survivin protein and mRNA expression and causes caspases activation and apoptosis in human bladder transitional-cell papilloma RT4 cells. *Biochem Biophys Res Commun* 2003; **312**: 1178-1184

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Tyrosine kinase of insulin-like growth factor receptor as target for novel treatment and prevention strategies of colorectal cancer

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Abstract

AIM: To investigate the antineoplastic potency of the novel insulin-like growth factor 1 receptor (IGF-1R) tyrosine kinase inhibitor (TKI) NVP-AEW541 in cell lines and primary cell cultures of human colorectal cancer (CRC).

METHODS: Cells of primary colorectal carcinomas were from 8 patients. Immunostaining and crystal violet staining were used for analysis of growth factor receptor protein expression and detection of cell number changes, respectively. Cytotoxicity was determined by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH). The proportion of apoptotic cells was determined by quantifying the percentage of sub-G1 (hypodiploid) cells. Cell cycle status reflected by the DNA content of the nuclei was detected by flow cytometry.

RESULTS: NVP-AEW541 dose-dependently inhibited the proliferation of colorectal carcinoma cell lines and primary cell cultures by inducing apoptosis and cell cycle arrest. Apoptosis was characterized by caspase-3 activation and nuclear degradation. Cell cycle was arrested at the G1/S checkpoint. The NVP-AEW541-mediated cell cycle-related signaling involved the inactivation of Akt and extracellular signal-regulated kinase (ERK) 1/2, the upregulation of the cyclin-dependent kinase inhibitors $p21^{Waf1/CIP1}$ and $p27^{Kip1}$, and the downregulation of the cell cycle promoter cyclin D1. Moreover, BAX was upregulated during NVP-AEW541-induced apoptosis, whereas Bcl-2 was downregulated. Measurement of LDH release showed that the antineoplastic effect of NVP-AEW541 was not due to general cytotoxicity of the compound. However, augmented antineoplastic effects were observed in combination treatments of NVP-AEW541 with either 5-FU, or the EGFR-antibody cetuximab, or the HMG-CoA-reductase inhibitor fluvastatin.

CONCLUSION: IGF-1R-TK inhibition is a promising novel approach for either mono- or combination treatment strategies of colorectal carcinoma and even for CRC chemoprevention.

Key words: Insulin-like growth factor receptor; Tyrosine kinase; Colorectal cancer; Apoptosis; Cell cycle arrest

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer-related deaths worldwide. Long-term survival of colorectal cancer is related to the stage of disease. Once distal metastases develop the prognosis is poor[1]. At least 40% of patients with colorectal cancer develop distal metastases and most of them die thereof[2]. Hence, innovative approaches are urgently needed to improve the treatment of advanced colorectal cancer.

There is evidence that insulin-like growth factor 1 receptor (IGF-1R) may be a promising protein for specific and targeted therapeutic approaches[3]. Several reports indicate that IGF-1R is overexpressed in the majority (> 90%) of colorectal carcinomas, most likely contributing to the aggressive growth characteristics of these tumors and the poor prognosis[4,5]. IGF-1R is a tetrameric transmembrane receptor tyrosine kinase (TK) composed of two α and two β subunits. The extracellular α subunit is responsible for ligand binding, whereas the β subunit consists of a transmembrane domain and a cytoplasmic tyrosine kinase domain[6,7]. The receptor is predominantly activated by IGF-I and II but can also be activated by insulin at a much lower affinity (500-1000 fold less). Ligand binding activates in-

trinsic tyrosine kinase activity, resulting in trans-β subunit autophosphorylation and stimulation of signaling cascades that include IRS-1/PI-3K/PKB/S6K and Grb2/Sos/Ras/MAPK pathways[8,9].

In general, both IGFs and IGF-1R are involved in the development and progression of several cancers[8-10]. Activation of IGF-1R by its ligands results in proliferation, survival, transformation, metastasis, and angiogenesis. Hence, abnormal or enhanced expression of IGFs and IGF-1R has been correlated with disease stage, reduced survival, metastasis development and de-differentiation of a broad variety of tumors. Obesity and diabetes are associated with an increased risk of colorectal cancer[11]. This effect seems to be due to alterations in the metabolism of endogenous hormones, including sex steroids, insulin and also activation of the IGF/IGF-receptor system which further supports the idea of the IGF/IGF-receptor system to be a promising target for colorectal cancer treatment and chemoprevention. Several studies have demonstrated the therapeutic potential of interfering with IGF-1R-mediated signaling in cancer cells *in vitro* and *in vivo*. These approaches include the use of antagonistic IGF-1R antibodies, IGF-1R antisense oligonucleotides, or IGF-1R siRNA[12-14]. Specific inhibition of IGF-1R TK activity appears to be another promising principle.

Recently, NVP-AEW541, an orally available low-molecular-weight pyrolo [2, 3-d] pyrimidine derivative, has been introduced as a potent and reversible inhibitor of IGF-1R tyrosine kinase activity[15]. NVP-AEW541 has been shown to be highly selective for IGF-1R-TK, as compared to both the closely related insulin receptor (InsR) and other tyrosine or serine/threonine kinases. Antitumor activity of NVP-AEW541 has already been demonstrated in fibrosarcomas and breast cancer[16]. IGFR-TK inhibition has not been evaluated for the treatment of colorectal cancer. Hence, in the present study we examined the antineoplastic potency of NVP-AEW541 both in human colorectal carcinoma cell lines and in primary cell cultures of human CRC, which shows that NVP-AEW541 potently inhibits colorectal cancer growth by inducing apoptosis and cell cycle arrest in human colorectal carcinoma cells.

MATERIALS AND METHODS

Cell lines and drugs

The human colorectal adenocarcinoma cell line HT29 was cultured in RPMI 1640 medium supplemented with 100 g/L fetal bovine serum, penicillin (100 kU/L), and streptomycin (100 mg/L). The human colorectal adenocarcinoma cell line HCT116 was grown in Dulbecco's minimal essential medium containing 100 g/L fetal bovine serum, penicillin (100 kU/L), and streptomycin (100 mg/L). Cells were kept in a humidified atmosphere (50 mL/L CO_2) at 37℃. Cells were incubated with culture medium containing NVP-AEW541 (Novartis, Basel, Switzerland). For combination treatment, cells were incubated simultaneously with NVP-AEW541 and one of the following drugs: 5-fluorouracil (Sigma, Deisenhofen, Germany), SN-38 (Rhone-Poulenc Rorer, Antony, France), cetuximab (Merck KgaA, Darmstadt, Germany) or fluvastatin (Calbiochem-Novabiochem, Bad Soden, Germany). Stock solutions (in DMSO, stored at -20℃) were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 1 mL/L.

Isolation and establishment of primary cell cultures from human colorectal cancers

Cells of primary colorectal carcinomas from 8 patients (5 males, 3 females, age range 74 ± 14 years) were isolated from endoscopically taken biopsies as previously described[17]. The human tumor material was used according to the standards set by the Ethical Committee of the Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany. Cell preparation was performed by incubation (30 min/RT) with a solution containing 0.5 g/L trypsin, 0.2 g/L EDTA and 1 g/L collagenase. The isolated human colorectal carcinoma cells were maintained in Earle's 199 medium supplemented with 200 g/L FBS, 2 mmol/L L-glutamine, 20 g/L Biotect protective medium, penicillin (100 kU/L), streptomycin (100 mg/L), 10 g/L amphotericin B, and incubated at 37℃ in a humidified atmosphere (50 mL/L CO_2).

Analysis of growth factor receptor expression

For analysis of growth factor receptor protein expression, cells were immunostained as previously described[18,19]. In brief, samples were fixed, permeabilized, and subsequently incubated with a polyclonal anti-EGFR or IGF-1R antibody (5 mg/L, Santa Cruz Biotechnology, Palo Alto, CA), or isotypic control rabbit IgG1 (DAKO, Hamburg, Germany). Cells were then incubated with a secondary FITC-labeled goat-anti rabbit IgG antibody (5 mg/L, BD Pharmingen, Heidelberg, Germany). Fluorescence was detected by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and analyzed using CellQuest software.

Determination of cell number

Cell number was evaluated by crystal violet staining as previously described[20]. In brief, cells in 96-well plates were fixed with 10 mL/L glutaraldehyde and then stained with 1 g/L crystal violet. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 2 g/L Triton X-100. Light extinction increasing linearly with the cell number was analyzed at 570 nm using an ELISA reader.

Determination of cytotoxicity

Cells were incubated with 0-10 μmol/L NVP-AEW541 for 1, 6, 12 and 24 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was measured using a colorimetric kit from Roche (Roche Diagnostics, Mannheim, Germany) as described previously[21,22]. Background release from untreated cells was subtracted. Maximum release was measured after adding 2 g/L Triton X-100 to untreated cells. For determinations, LDH assay reagent was added to sample supernatants and incubated for 30 min at room temperature in dark. Absorbance was measured at 490 nm (reference wavelengh 690 nm).

Detection of apoptosis

Preparation of cell lysates and determination of caspase-3 activity were performed as previously described[23]. The activity of caspase-3 was calculated from the cleavage of the fluorogenic substrate DEVD-AMC (Calbiochem-Novabiochem, Bad Soden, Germany). The proportion of apoptotic cells was determined by quantifying the percentage of sub-G1 (hypodiploid) cells after flow cytometric analysis of isolated propidium iodide-stained nuclei[24].

Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelov and Christensen[25]. Cells were trypsinized, washed, and the nuclei were isolated using CycleTest PLUS DNA Reagent Kit (Becton Dickinson). DNA was stained with propidium iodide according to the manufacturer's instructions. DNA content of the nuclei was detected by flow cytometry and analyzed using CellFit software (Becton Dickinson).

Western blotting

Western blotting was performed as previously described[23]. Blots were blocked in 50 g/L non-fat dry milk for 1 h, and then incubated at 4℃ overnight with anti-phospho-Akt, Akt (both 1:1000, Cell Signaling, Beverly, MA), Bax (1:1000, Santa Cruz Biotechnology, CA), Bcl-2 (1:200, Novo Castra Laboratories, Newcastle upon Tyne, UK), COX-2 (1:200), cyclin D1 (1:100), $p21^{Waf1/CIP1}$ (1:200), IGF-1R (1:200), phospho-ERK1/2, ERK1/2 (1:1000, Santa Cruz Biotechnology, CA), or $p27^{Kip1}$ (1:2500, Becton Dickinson). β-actin (1:5000, Sigma) served as a loading control.

Statistical analysis

If not stated otherwise, means of four independent experiments ± SE were shown. Individual drug treatment was compared by the unpaired, two-tailed Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of IGF-1R in colorectal carcinoma cells

Expression of IGF-1R and epidermal growth factor receptor (EGFR) was investigated in human colorectal carcinoma cells. Protein expression of IGF-1R was detected in both cell lines. In addition, expression of EGFR protein was detected in both cell lines (Figure 1A). IGF-1R protein expression of NVP-AEW541-treated colorectal cancer cells was determined by Western blotting. HT-29 cells incubated with NVP-AEW541 (0-10 μmol/L) for 48 h did not abolish the expression of IGF-1R. By contrast even after treatment with 10 μmol/L of NVP-AEW541, a robust expression of IGF-1R protein could still be observed (Figure 1B). IGF-1R and EGFR expression was confirmed in the investigated 8 primary colorectal cancer cultures by RT-PCR using established primers (not shown)[18,22].

NVP-AEW541-induced growth inhibition of colorectal carcinoma cells

Cell number changes caused by IGF-1R-TK inhibition

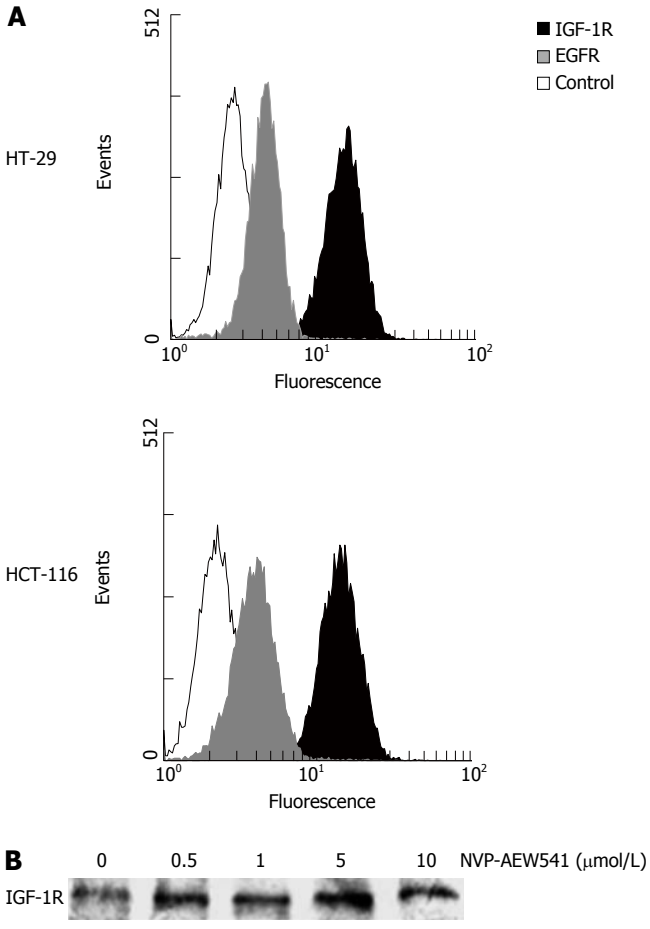

Figure 1 Flow cytometric analysis of IGF-1R and EGFR protein expression in HT-29 (**A**) and HCT-116 (**B**) cells. Cells were stained with antibodies against either IGF-1R (black areas) or EGFR (grey areas). Black lines: isotypic controls.

with NVP-AEW541 were studied by crystal violet assays. NVP-AEW541 time- and dose-dependently inhibited the growth of HT-29 and HCT-116 cells (Figure 2A and B). The IC_{50} values of NVP-AEW541 were 1.7 ± 0.4 μmol/L (HT-29) and 2.5 ± 0.4 μmol/L (HCT-116), as determined after 4 d of incubation.

In line with our findings in permanent cell lines, NVP-AEW541 treatment (0-5 μmol/L) reduced the cell number of primary cultures of human colorectal carcinomas in a dose-dependent manner. After 3 d of incubation a cell number reduction of 47.3% ± 2.4% was detected by direct cell counting in six NVP-AEW541-sensitive primary culture preparations. Two out of the investigated 8 primary cultures displayed only a weak growth inhibition of 12% ± 4%. In treatment-sensitive primary cultures NVP-AEW541 also altered the morphology of the remaining cells, which appeared shrunken and flat. Propidium iodide-positive staining of primary culture cells revealed that NVP-AEW541 treatment led to a loss of cell membrane integrity indicating cell death or that these cells were in the process of dying, respectively (Figure 2C).

Antineoplastic potency of NVP-AEW541 in combination with cytostatics, cetuximab or the HMG-CoA reductase inhibitor fluvastatin

To test whether combination treatment of NVP-AEW541

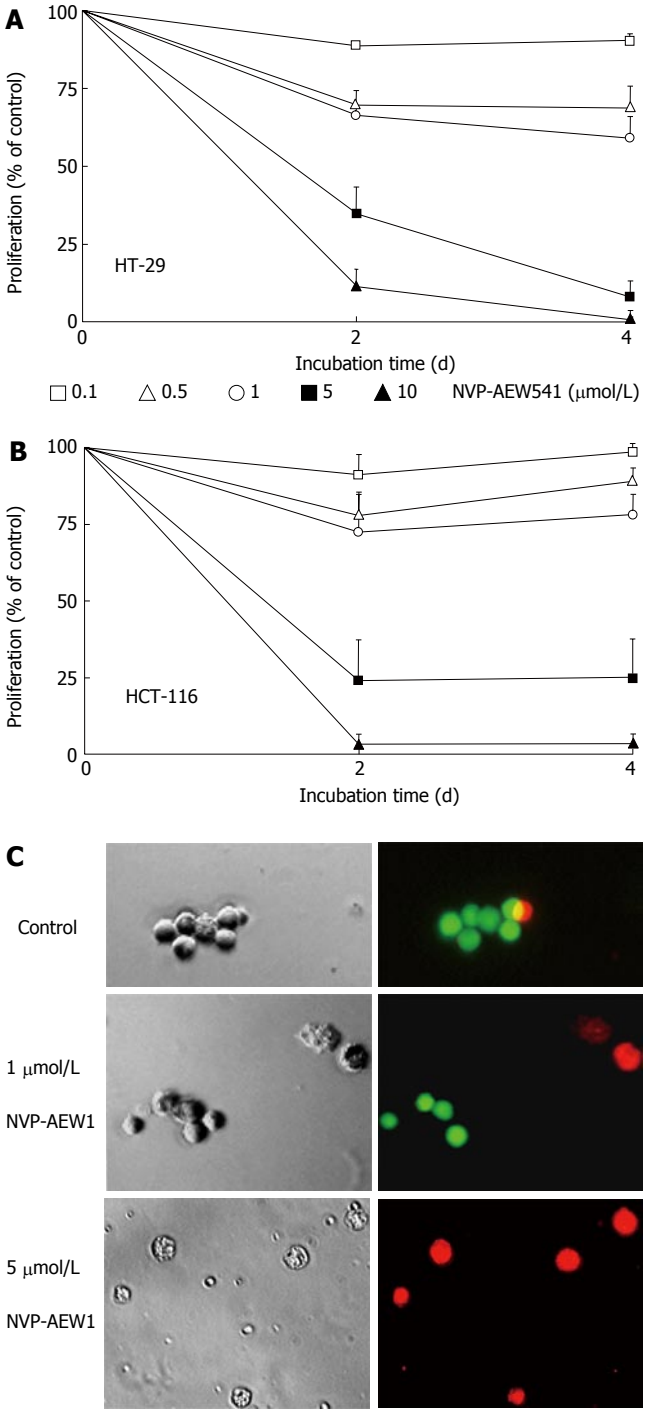

Figure 2 Effects of NVP-AEW541 on HT-29 (**A**) and HCT-116 (**B**) cell growth as well as induction of cell death and morphological changes of isolated primary colorectal cancer cells (**C**). After 4 d of incubation with rising concentrations of NVP-AEW541, the number of HT-29 (**A**) and HCT-116 (**B**) cells decreased by > 95%, as determined by crystal violet staining (mean ± SE, n = 4). In both cell lines statistical significance (P < 0.05) of growth inhibition by NVP-AEW541 was shown for concentrations of 0.5-10 μmol/L. After 3 d of incubation with 0-5 μmol/L NVP-AEW541, the induction of cell death and morphological changes of isolated primary colorectal cancer cells was determined by Live/Dead-fluorescence microscopy (**C**). Viable cells are stained green, while cells with impaired cell membrane appear red. Phase-contrast images and corresponding fluorescence micrographs of a representative preparation (out of 6 NVP-AEW541-sensitive primary cell cultures) are depicted.

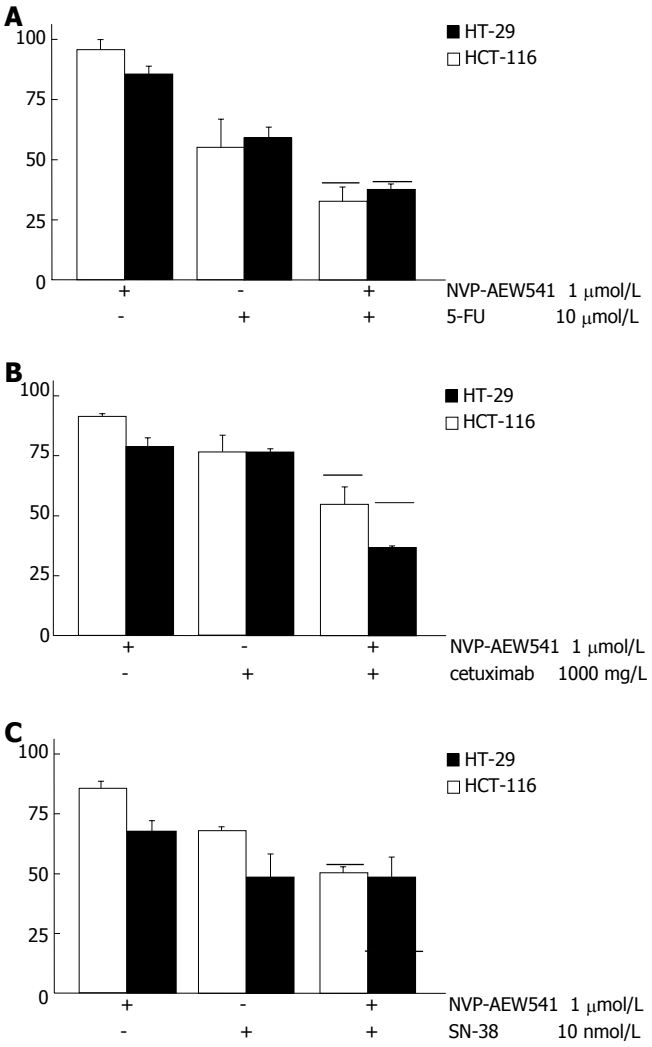

Figure 3 Augmented growth inhibition of combination treatment with NVP-AEW541 plus either 5-FU (**A**), or SN-38 (**B**) or cetuximab (**C**) (mean ± SE, n = 4-6). **A**: Combination treatment with sub-IC_{50} concentrations of NVP-AEW541 plus 5-FU led to synergistic growth inhibition of colorectal cancer cells; **B**: Combination treatment with sub-IC_{50} concentrations of NVP-AEW541 plus the humanized EGFR-antibody cetuximab resulted in slightly over-additive antiprolifertive effects; **C**: Co-treatment with sub-IC_{50} concentrations of NVP-AEW541 and SN-38 resulted in additive growth inhibition of HCT-116 cells, while no additive growth inhibition was detected in HT-29 cells. Black bars indicate the values of the calculated additive growth inhibition. Data are given as percentage of untreated controls.

with either 5-fluorouracil (5-FU), SN-38, or whether the humanized monoclonal anti-EGFR antibody cetuximab may lead to additive antiproliferative effects, HT29 and HCT-116 cells were co-treated for 96 h with sub-IC_{50} concentrations of NVP-AEW541 (1 μmol/L) plus either 5-FU (10 μmol/L), or cetuximab (1000 mg/L), or SN-38 (10 nmol/L). In both colorectal carcinoma cell models combination treatment with NVP-AEW541 plus either 5-FU (Figure 3A) or cetuximab (Figure 3B) resulted in additive or even over-additive growth inhibitory effects. When NVP-AEW541 was combined with SN-38, additive antiproliferative effects were only observed in HCT-116 cells (Figure 3C). We additionally investigated the antiproliferative potency of the HMG-CoAR inhibitor fluvastatin, either alone or in combination with NVP-AEW541 in colorectal carcinoma cells (Figure 4). Fluvastatin (0-50 μmol/L) caused a dose-dependent growth inhibition of more than 80% both in HCT-116 cells and in HT-29 cells. Combinations of sub-IC_{50} concentrations of fluvastatin and NVP-AEW541 resulted in an additive growth inhibi-

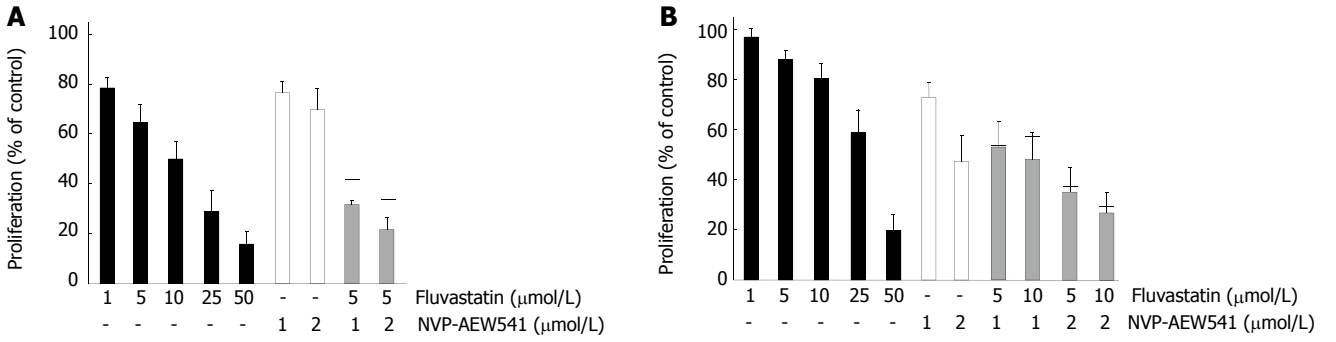

Figure 4 Additive growth inhibition by NVP-AEW541 plus fluvastatin (mean ± SE, *n* = 3-5). Fluvastatin (1-50 μmol/L) induced a dose-dependent growth inhibition of HCT-116 (**A**) and HT-29 (**B**) cells by > 80% when applied for 3 d. Moreover, combination treatment with sub-IC_{50} concentrations of fluvastatin and NVP-AEW541 led to (over-)additive growth inhibition after 3 d of treatment. Black bars indicate the values of the calculated additive growth inhibition.

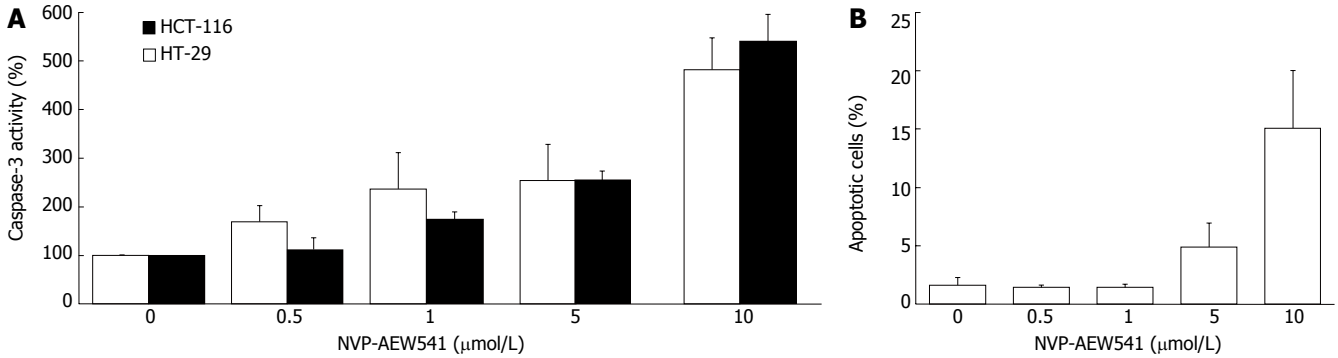

Figure 5 Apoptosis induction by NVP-AEW541 (mean ± SE, *n* = 4). **A**: After HT-29 and HCT-116 cells were incubated with 0.5-10 μmol/L NVP-AEW541 for 24 h, a dose-dependent induction in caspase-3 activation was observed; **B**: After 72 h of NVP-AEW541 treatment an increased proportion of apoptotic cells measured as subdiploidy was observed in HT-29 cells. $^{a}P < 0.05$ *vs* untreated controls.

tion of either colorectal cancer model.

NVP-AEW541-induced apoptosis in colorectal cancer cells

NVP-AEW541 dose-dependently induced a significant increase of caspase-3 activity after 24 h of incubation (Figure 5A). Compared to control cells, an increase of up to 500% was observed. The dose-dependent induction of apoptosis by NVP-AEW541 became also apparent by flow cytometrically monitoring nuclear degradation after 72 h of treatment (Figure 5B).

NVP-AEW541-induced cell cycle arrest in colorectal carcinoma cells.

To test whether an induction of cell cycle arrest contributed to the antiproliferative potency of NVP-AEW541 in colorectal carcinoma cells, we performed cell cycle analyses. NVP-AEW541 dose-dependently arrested HT-29 and HCT-116 cells in the G1/G0 phase of the cell cycle after 24 h of treatment, thereby decreasing the proportion of cells in the S and G2/M phases (Figure 6).

Cytoxicity of NVP-AEW541

Cytotoxicity of NVP-AEW541 was determined by measuring LDH release. HT29 and HCT-116 cells incubated with 1-10 μmol/L NVP-AEW541 for 1, 6 or 12 h did not result in a measurable increase in LDH release, indicating that NVP-AEW541 did not directly affect cell membrane integrity. After 24 h of incubation a slight but not significant increase in LDH-release of about 3% was observed at 10 μmol/L NVP-AEW541, indicating that even at high concentrations NVP-AEW541 did not cause immediate necrotic/cytotoxic effects in colorectal cancer cells (data not shown).

NVP-AEW541-induced modulation of cell cycle and apoptosis-related signaling molecules

The effects of NVP-AEW541 on the phosphorylation of both ERK1/2 and its upstream regulator Akt/PKB were investigated to elucidate the signaling pathways modulated by IGF-1R-TK inhibition. NVP-AEW541 treatment dose-dependently decreased the phosphorylation of both mitogenic and antiapoptotic ERK1/2 MAPK as well as Akt/PKB (Figure 7A). Next, the expression of cell cycle-related proteins was investigated to explore the pathway downstream of NVP-AEW541-induced dephosphorylation of Akt/PKB and ERK1/2, known to be causative for cell cycle arrest. NVP-AEW541 decreased the expression of cyclin D1 but increased the expression of the CDK inhibitors $p21^{Waf1/CIP1}$ and $p27^{Kip1}$. These data suggest that NVP-AEW541-induced cell cycle arrest was mediated by $p21^{Waf1/CIP1}$ and $p27^{Kip1}$ induction, resulting in a decrease of cyclin D1.

To survey the proapoptotic signaling pathways modulated by IGF-1R-TK inhibition with NVP-AEW541 in CRC

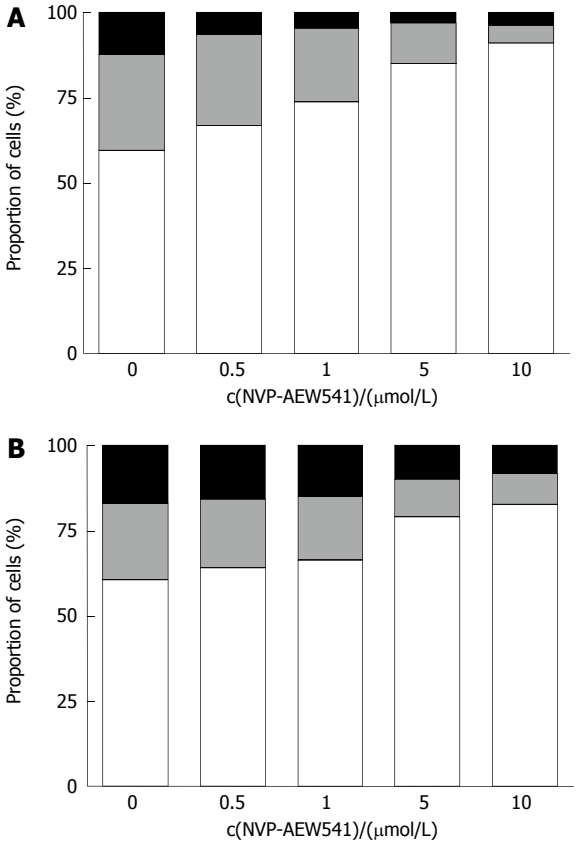

Figure 6 Effects of NVP-AEW541 on the cell cycle of colorectal carcinoma cells (means ± SE, $n = 4$). After 24 h incubation with NVP-AEW541, a dose-dependent accumulation of HT-29 (**A**) and HCT-116 (**B**) cells was observed in the G0/G1 phase of the cell cycle (white bars). Proportion of cells in the S and G2/M phase (grey and black bars) decreased. $^aP < 0.05$ *vs* untreated controls.

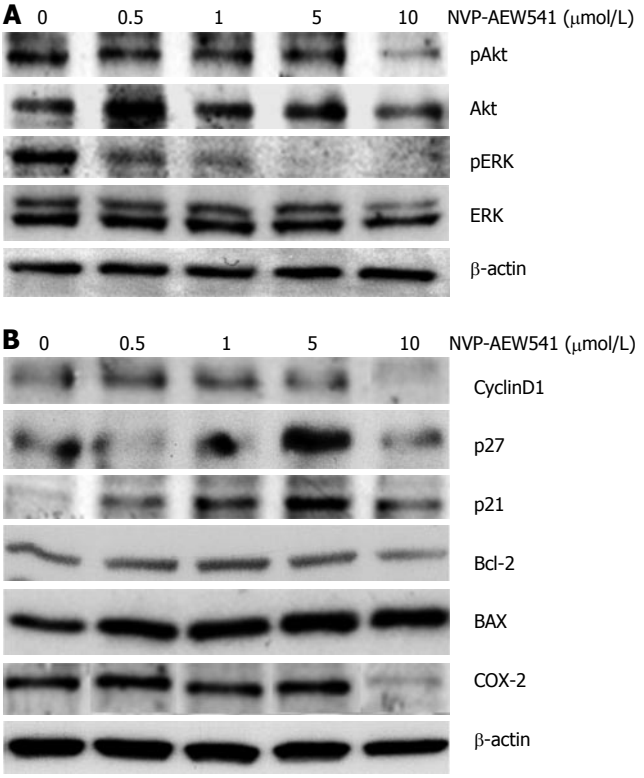

Figure 7 Effects of NVP-AEW541 on the expression and phosphorylation of apoptosis- and cell cycle- related proteins. Modulation of protein phosphorylation or protein expression by NVP-AEW541 treatment was analyzed by Western blotting. **A**: NVP-AEW541 treatment (24 h) induced a dose-dependent dephosphorylation of mitogenic ERK1/2; **B**: NVP-AEW541 dose-dependently increased the expression of the proapoptotic Bax protein, while the expression of the antiapoptotic protein Bcl-2 slightly decreased. The cell cycle promoter cyclin D1 was down-regulated by NVP-AEW541 treatment, while the cell cycle inhibitors $p21^{Waf1/CIP1}$ and $p27^{Kip1}$ were up-regulated. Moreover, COX-2 expression was down-regulated by NVP-AEW541. Expression of β-actin was used as a loading control.

cells, we also investigated the effects of NVP-AEW541 on the expression pattern of Bcl-2 and Bax. Treatment with NVP-AEW541 dose-dependently increased the expression of the proapoptotic Bax protein, while the expression of the antiapoptotic protein Bcl-2 slightly decreased. Finally, it was proved that NVP-AEW541 could downregulate the expression of cyclooxygenase 2 (COX-2) in colorectal cancer cells (Figure 7B).

DISCUSSION

Treatment options for advanced colorectal cancer (CRC) are unsatisfactory. Thus, there is a strong need for effective novel treatment strategies of CRC. A novel approach for CRC treatment may be the interruption of IGF/IGF-receptor signaling system, which is known to have strong stimulatory effects on cancer cell growth. The protective and mitogenic effects of the IGF/IGF-receptor system involve the constitutive activation of antiapoptotic proteins as well as cell cycle promoting signaling. A tight association between IGF-receptor signaling and regulation of cell growth and apoptosis in CRCs which commonly overexpress IGF-receptors has been described[4,5,26].

Most fast growing cancers produce and release growth factors and thereby auto-stimulate their growth. This also holds true for CRC in which epidermal growth factor (EGF), transforming growth factor α (TGF-α), insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) are produced and secreted to promote CRC growth[27]. Therefore, the antiproliferative effects of the novel IGF-1R tyrosine kinase inhibitor NVP-AEW541 were investigated under serum-containing conditions (e.g. in the presence of growth factors like EGF, IGF and TGF-α). Our study demonstrated that inhibition of IGF-1R tyrosine kinase activity by NVP-AEW541 might be suitable for novel targeted therapy of CRC. By abrogating the protective and mitogenic effects of IGF-R signaling, CRC cell growth was potently inhibited by NVP-AEW541. The antineoplastic effects of NVP-AEW541 were based on a pronounced induction of cell cycle arrest and apoptosis. Accompanying or dose-limiting cytotoxicity was not observed, underlining the specific mode of action of the drug. Our findings are in line with recent observations on IGF-R inhibition in other cancer models. Although applying other approaches for IGF-R inhibition, these studies have also shown the strong antineoplastic potency of the interruption of IGF-R signaling without dose-limiting toxicity in several other cancer models[16,28,29].

Re-initialisation of apoptosis and induction of cell cycle arrest are valuable features for a successful anti-cancer agent. On the one hand, apoptotic cell death is not accompanied with undesired immunological reactions, which occur upon treatment with unspecific and cytotoxic

agents. On the other hand, apoptosis-sensitization and cell cycle arresting effects offer possibilities for powerful combination treatments. In this respect, we investigated combination treatments of NVP-AEW541 and clinically relevant cytostatics with different modes of action. The tested combinations of DNA strand-breaking pyrimidine analog 5-fluorouracil (5-FU) and topoisomerase-I inhibitor SN-38, except for the combination of NVP-AEW541 and SN-38 in HT29 cells, resulted in additive antiproliferative effects. The additive antineoplastic potency appeared to be independent of the mode of action of the respective cytostatic drug. Our data support the notion that NVP-AEW541 is a promising new agent for potentiation of antitumor efficay of the established cytostatic CRC treatment.

It has been pointed out that the antiapoptotic potency of IGF/IGFR-signaling might interfere with strategies that target other tyrosine kinases such as EGFR-TK. Hence, the antineoplastic potency of EGFR blockade may well be underestimated when examined under conditions where IGF-1R is fully functional. IGF-1R is capable of transactivating EGFR-TK and abrogating the antiproliferative effects of EGFR-antibody treatment[30-32]. As the CRC cell models used in this study were shown to express both IGF-1R and EGFR, we studied the effects of targeting both growth factor receptors by combination treatment. Treating CRC cells with sub-IC_{50} concentrations of both NVP-AEW541 and humanized anti-EGFR-antibody cetuximab enhanced the antiproliferative effect as compared to the effect of either agent alone. Thus, NVP-AEW541 qualifies as a promising substance for combination treatment strategies to overcome the compensatory effects of mitogenic crosstalks between IGF-1R and EGFR in CRC.

Drug resistance is one of the major problems of chemotherapy. Potential mechanisms of drug resistance include the activation of Ras/Raf/Mek/ERK signal transduction cascade and the increase of cholesterol levels in cancer cells, both being controlled by isoprenoids[33]. The production of isoprenoids is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) which may therefore be a rational molecular target for innovative antineoplastic treatment of colorectal cancer. Fluvastatin is an effective inhibitor of HMG-CoAR and has already been shown to inhibit tumor cell growth[34]. In the present study, we have demonstrated the antineoplastic effect of fluvastatin alone and in combination with NVP-AEW541 in colorectal cancer cells and the antiproliferative effect of NVP-AEW541 augmented by fluvastatin, suggesting that combining NVP-AEW541 and fluvastatin may be a promising approach for dual targeting treatment strategies in colorectal cancer disease.

The mechanisms underlying the antiproliferative action of NVP-AEW541 in colorectal cancer cells were further characterized. NVP-AEW541 induced cell cycle arrest in the G1/G0-phase in both CRC cell lines, suggesting that the drug acts at the G1/S checkpoint. A G1/S cell cycle arrest induced by inhibition of IGFR signaling has been described in other fast growing cancers[35,36]. Moreover, we observed a definite rise in apoptotic cells after treatment with NVP-AEW541. While the induction of apoptosis is a well-known effect occurring upon inhibition of IGFR-signaling, the underlying mechanisms have been poorly characterized[37-39]. Our present results suggest that activation of caspase-3 is involved in NVP-AEW541-induced apoptosis of CRC cells. The proapoptotic protein BAX was upregulated during NVP-AEW541-induced apoptosis, while Bcl-2, the protective and antiapoptotic counterpart of Bax, was downregulated. Our findings suggest that involvement of mitochondrial pathways leads to NVP-AEW541-mediated apoptosis.

As activated IGF-1R induces the Ras-Raf-MEK-ERK signaling pathway with subsequent induction of cyclin D1 expression, we analyzed NVP-AEW541-induced changes of Akt/PKB, ERK1/2 activity and $p21^{Waf1/CIP1}$, $p27^{Kip1}$ as well as cyclin D1 expression as previously described[40]. In agreement with previous observations in non-colorectal tumor models[16], we found that NVP-AEW541 could dephosphorylate IGF-1R as well as ERK1/2 MAPK and AKT/PKB of colorectal cancer cells.

IGF-IR has at least three survival signals that are able to protect cancer cells from apoptosis, namely PI-33K/AKT and MAPK/ERK signaling pathways, and a third one that results in the mitochondrial translocation of Raf 1, the so called 14-3-3 pathway[41]. Simultaneous inactivation of two of these pathways is required to inhibit IGF-1R capacity of protecting cells from apoptotic injuries[42]. By showing the simultaneous inactivation of AKT/PKB and ERK1/2 MAPK by NVP-AEW541, we hypothezise that blockade of these two survival pathways is directly involved in the successful inhibition of colorectal cancer cell growth by NVP-AEW541.

Defective function of cell cycle regulators is a main cause for tumor development and progression. For example, the cell cycle promoter cyclin D1 is frequently overexpressed in CRC. Successful therapeutic strategies have to balance or bypass the impaired signaling. In the present study, NVP-AEW541 treatment raised the expression of the cell cycle-inhibiting molecules $p21^{Waf1/CIP1}$ and $p27^{Kip1}$, while it decreased the expression of cyclin D1. Finally, the expression of COX-2 which is known to be upregulated during colorectal carcinogenesis and plays an important role in colorectal cancer growth, was suppressed by IGFR-TK inhibition as previously described[43]. Thus, NVP-AEW541-induced COX-2 suppression may well contribute to its antineoplastic potency in colorectal cancer. Since both COX-2 and IGFR are up-regulated during colorectal carcinogenesis, NVP-AEW541 appears to be a promising chemopreventive agent in patients at risk for CRC.

Primary cell cultures of human colorectal cancers were established as a tool to design a rational individual medical treatment of an individual patient. The primary goal was to study NVP-AEW541's antineoplastic potency in a bench to bedside approach, as permanent cell lines may represent well-suited but nevertheless non-representative models of colorectal cancers. Moreover, chemosensitivity testing of primary cultures was performed to establish a new method for predicting the response of an individual patient to a certain drug. Attempts to predict individual responses have already been undertaken for breast cancer and colorectal cancer, respectively[44,45]. Such an approach

may pave the way to an individualized medical treatment of cancer patients. The importance of predictive testing is further supported by our finding that two out of the tested eight primary cell cultures showed only a weak response to NVP-AEW541 treatment. Nevertheless, NVP-AEW541 is a promising compound for future colorectal cancer treatment, as 75% of the investigated primary colorectal cancers could be effectively treated with the drug.

In conclusion, the IGFR-TK inhibitor NVP-AEW541 potently inhibits the growth of human colorectal cancer cells by inducing both cell cycle arrest and apoptosis without eliciting unspecific cytotoxicity. Furthermore, the compound is well-suited for combination treatment approaches. Thus, inhibition of IGFR-signaling by NVP-AEW541 is a promising targeted anticancer strategy for colorectal carcinoma and should be tested in future clinical trials. Moreover, investigations should be pursued to modulate the IGF/IGFR system as a possible means of chemoprevention of colorectal cancer in patients at risk.

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REFERENCES

1 **Weitz J**, Koch M, Debus J, Höhler T, Galle PR, Büchler MW. Colorectal cancer. *Lancet* 2005; **365**: 153-165
2 **Rougier P**, Mitry E. Epidemiology, treatment and chemoprevention in colorectal cancer. *Ann Oncol* 2003; **14** Suppl 2: ii3-ii5
3 **Larsson O**, Girnita A, Girnita L. Role of insulin-like growth factor 1 receptor signalling in cancer. *Br J Cancer* 2005; **92**: 2097-2101
4 **Hakam A**, Yeatman TJ, Lu L, Mora L, Marcet G, Nicosia SV, Karl RC, Coppola D. Expression of insulin-like growth factor-1 receptor in human colorectal cancer. *Hum Pathol* 1999; **30**: 1128-1133
5 **Weber MM**, Fottner C, Liu SB, Jung MC, Engelhardt D, Baretton GB. Overexpression of the insulin-like growth factor I receptor in human colon carcinomas. *Cancer* 2002; **95**: 2086-2095
6 **Ullrich A**, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell* 1990; **61**: 203-212
7 **van der Geer P**, Hunter T, Lindberg RA. Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol* 1994; **10**: 251-337
8 **Wang Y**, Sun Y. Insulin-like growth factor receptor-1 as an anti-cancer target: blocking transformation and inducing apoptosis. *Curr Cancer Drug Targets* 2002; **2**: 191-207
9 **Baserga R**. The contradictions of the insulin-like growth factor 1 receptor. *Oncogene* 2000; **19**: 5574-5581
10 **Schoen RE**, Weissfeld JL, Kuller LH, Thaete FL, Evans RW, Hayes RB, Rosen CJ. Insulin-like growth factor-I and insulin are associated with the presence and advancement of adenomatous polyps. *Gastroenterology* 2005; **129**: 464-475
11 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638
12 **Scotlandi K**, Benini S, Nanni P, Lollini PL, Nicoletti G, Landuzzi L, Serra M, Manara MC, Picci P, Baldini N. Blockage of insulin-like growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice. *Cancer Res* 1998; **58**: 4127-4131
13 **Shapiro DN**, Jones BG, Shapiro LH, Dias P, Houghton PJ. Antisense-mediated reduction in insulin-like growth factor-I receptor expression suppresses the malignant phenotype of a human alveolar rhabdomyosarcoma. *J Clin Invest* 1994; **94**: 1235-1242
14 **Salisbury AJ**, Macaulay VM. Development of molecular agents for IGF receptor targeting. *Horm Metab Res* 2003; **35**: 843-849
15 **Scotlandi K**, Manara MC, Nicoletti G, Lollini PL, Lukas S, Benini S, Croci S, Perdichizzi S, Zambelli D, Serra M, García-Echeverría C, Hofmann F, Picci P. Antitumor activity of the insulin-like growth factor-I receptor kinase inhibitor NVP-AEW541 in musculoskeletal tumors. *Cancer Res* 2005; **65**: 3868-3876
16 **García-Echeverría C**, Pearson MA, Marti A, Meyer T, Mestan J, Zimmermann J, Gao J, Brueggen J, Capraro HG, Cozens R, Evans DB, Fabbro D, Furet P, Porta DG, Liebetanz J, Martiny-Baron G, Ruetz S, Hofmann F. In vivo antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell* 2004; **5**: 231-239
17 **Höpfner M**, Maaser K, Theiss A, Lenz M, Sutter AP, Kashtan H, von Lampe B, Riecken EO, Zeitz M, Scherübl H. Hypericin activated by an incoherent light source has photodynamic effects on esophageal cancer cells. *Int J Colorectal Dis* 2003; **18**: 239-247
18 **Höpfner M**, Sutter AP, Gerst B, Zeitz M, Scherübl H. A novel approach in the treatment of neuroendocrine gastrointestinal tumours. Targeting the epidermal growth factor receptor by gefitinib (ZD1839). *Br J Cancer* 2003; **89**: 1766-1775
19 **Sutter AP**, Maaser K, Höpfner M, Barthel B, Grabowski P, Faiss S, Carayon P, Zeitz M, Scherübl H. Specific ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in human esophageal cancer cells. *Int J Cancer* 2002; **102**: 318-327
20 **Gillies RJ**, Didier N, Denton M. Determination of cell number in monolayer cultures. *Anal Biochem* 1986; **159**: 109-113
21 **Decker T**, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* 1988; **115**: 61-69
22 **Höpfner M**, Sutter AP, Beck NI, Barthel B, Maaser K, Jockers-Scherübl MC, Zeitz M, Scherübl H. Meta-iodobenzylguanidine induces growth inhibition and apoptosis of neuroendocrine gastrointestinal tumor cells. *Int J Cancer* 2002; **101**: 210-216
23 **Höpfner M**, Sutter AP, Huether A, Schuppan D, Zeitz M, Scherübl H. Targeting the epidermal growth factor receptor by gefitinib for treatment of hepatocellular carcinoma. *J Hepatol* 2004; **41**: 1008-1016
24 **Sutter AP**, Maaser K, Grabowski P, Bradacs G, Vormbrock K, Höpfner M, Krahn A, Heine B, Stein H, Somasundaram R, Schuppan D, Zeitz M, Scherübl H. Peripheral benzodiazepine receptor ligands induce apoptosis and cell cycle arrest in human hepatocellular carcinoma cells and enhance chemosensitivity to paclitaxel, docetaxel, doxorubicin and the Bcl-2 inhibitor HA14-1. *J Hepatol* 2004; **41**: 799-807
25 **Vindeløv L**, Christensen IJ. An integrated set of methods for routine flow cytometric DNA analysis. *Methods Cell Biol* 1990; **33**: 127-137
26 **Reinmuth N**, Fan F, Liu W, Parikh AA, Stoeltzing O, Jung YD, Bucana CD, Radinsky R, Gallick GE, Ellis LM. Impact of insulin-like growth factor receptor-I function on angiogenesis, growth, and metastasis of colon cancer. *Lab Invest* 2002; **82**: 1377-1389
27 **Barozzi C**, Ravaioli M, D'Errico A, Grazi GL, Poggioli G, Cavrini G, Mazziotti A, Grigioni WF. Relevance of biologic markers in colorectal carcinoma: a comparative study of a

broad panel. *Cancer* 2002; **94**: 647-657
28 **Arteaga CL**, Kitten LJ, Coronado EB, Jacobs S, Kull FC Jr, Allred DC, Osborne CK. Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. *J Clin Invest* 1989; **84**: 1418-1423
29 **Andrews DW**, Resnicoff M, Flanders AE, Kenyon L, Curtis M, Merli G, Baserga R, Iliakis G, Aiken RD. Results of a pilot study involving the use of an antisense oligodeoxynucleotide directed against the insulin-like growth factor type I receptor in malignant astrocytomas. *J Clin Oncol* 2001; **19**: 2189-2200
30 **Gilmore AP**, Valentijn AJ, Wang P, Ranger AM, Bundred N, O'Hare MJ, Wakeling A, Korsmeyer SJ, Streuli CH. Activation of BAD by therapeutic inhibition of epidermal growth factor receptor and transactivation by insulin-like growth factor receptor. *J Biol Chem* 2002; **277**: 27643-27650
31 **Ahmad T**, Farnie G, Bundred NJ, Anderson NG. The mitogenic action of insulin-like growth factor I in normal human mammary epithelial cells requires the epidermal growth factor receptor tyrosine kinase. *J Biol Chem* 2004; **279**: 1713-1719
32 **Lu Y**, Zi X, Pollak M. Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. *Int J Cancer* 2004; **108**: 334-341
33 **Jakobisiak M**, Golab J. Potential antitumor effects of statins (Review). *Int J Oncol* 2003; **23**: 1055-1069
34 **Paragh G**, Kertai P, Kovacs P, Paragh G, Fülöp P, Foris G. HMG CoA reductase inhibitor fluvastatin arrests the development of implanted hepatocarcinoma in rats. *Anticancer Res* 2003; **23**: 3949-3954
35 **Mitsiades CS**, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, Hideshima T, Chauhan D, Joseph M, Libermann TA, García-Echeverría C, Pearson MA, Hofmann F, Anderson KC, Kung AL. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. *Cancer Cell* 2004; **5**: 221-230
36 **Baserga R**. Oncogenes and the strategy of growth factors. *Cell* 1994; **79**: 927-930
37 **Camirand A**, Pollak M. Co-targeting IGF-1R and c-kit: synergistic inhibition of proliferation and induction of apoptosis in H 209 small cell lung cancer cells. *Br J Cancer* 2004; **90**: 1825-1829
38 **Baserga R**, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. *Int J Cancer* 2003; **107**: 873-877
39 **LeRoith D**, Roberts CT Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 2003; **195**: 127-137
40 **Huynh H**, Do PT, Nguyen TH, Chow P, Tan PH, Quach TH, Van T, Soo KC, Tran E. Extracellular signal-regulated kinase induces cyclin D1 and Cdk-2 expression and phosphorylation of retinoblastoma in hepatocellular carcinoma. *Int J Oncol* 2004; **25**: 1839-1847
41 **Thomas D**, Guthridge M, Woodcock J, Lopez A. 14-3-3 protein signaling in development and growth factor responses. *Curr Top Dev Biol* 2005; **67**: 285-303
42 **Salatino M**, Schillaci R, Proietti CJ, Carnevale R, Frahm I, Molinolo AA, Iribarren A, Charreau EH, Elizalde PV. Inhibition of in vivo breast cancer growth by antisense oligodeoxynucleotides to type I insulin-like growth factor receptor mRNA involves inactivation of ErbBs, PI-3K/Akt and p42/p44 MAPK signaling pathways but not modulation of progesterone receptor activity. *Oncogene* 2004; **23**: 5161-5174
43 **Brown JR**, DuBois RN. COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol* 2005; **23**: 2840-2855
44 **Shukla GS**, Krag DN. Selection of tumor-targeting agents on freshly excised human breast tumors using a phage display library. *Oncol Rep* 2005; **13**: 757-764
45 **Mori S**, Kunieda K, Sugiyama Y, Saji S. Prediction of 5-fluorouracil and cisplatin synergism for advanced gastrointestinal cancers using a collagen gel droplet embedded culture. *Surg Today* 2003; **33**: 577-583

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COLORECTAL CANCER

Hemoglobin induces colon cancer cell proliferation by release of reactive oxygen species

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Abstract

AIM: To study whether hemoglobin could amplify colon cancer cell proliferation *via* reactive oxygen species (ROS) production.

METHODS: Colon cancer cell line HT-29 was grown in the conventional method using RPMI1640 media. The viability of the cells was measured using the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay after adding hemoglobin. We determined reactive oxygen species levels to be indicators of oxidative stress in HT 29 cell lines with and without hemoglobin and/or 5-fluorouracil (5-FU), 5'-deoxy-5-fluorouridine (5-DFUR) using fluorometric dichlorofluorescin diacetate (DCFH-DA) assay.

RESULTS: Cellular proliferation was increased with hemoglobin in a concentration-dependent manner. A significant increment on ROS levels was found in HT 29 cells following hemoglobin incubation. The cytotoxic effects of 5-FU and 5-DFUR were significantly blunted by administration of hemoglobin. There was a slight increase of peroxiredoxin 1, superoxide dismutase 1 concentration according to different hemoglobin concentrations.

CONCLUSION: Hemoglobin has a cellular proliferative effect on HT-29 colon cancer cell line by production of ROS. Also, hemoglobin abates cytotoxic effects of chemotherapeutic agents such as 5-FU and 5-DFUR.

Key words: Colon cancer; Hemoglobin; Reactive oxygen species

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INTRODUCTION

Colon cancer is an important public health issue[1]. There are nearly one million cases of colon cancer diagnosed worldwide each year. The increasing trend of this cancer is prominent in Asian countries, including Korea[2,3]. Until the present day, scientists have made an intensive effort to find a provocative factor of this major cancer. Many epidemiologic studies indicate that a western style diet is associated with a high incidence of colon cancer[4-6]. An especially high protein consumption as in the western-style diet is regarded as a major factor in inducing colon cancer. There is consistent evidence that high meat consumption, in particular red meat, confers an increased risk of this cancer. However, recent large prospective epidemiologic studies that hypothesized a strong relationship between red meat consumption and colorectal cancer development have revealed inconsistent results.

Hemoglobin is a complex of heme and globin, which contribute an important role of oxygen delivery processes to individual tissues[7]. Intake of these particular molecules contributes nutritional buildup as an iron and protein supplementation. Hemoglobin inside food is already in an oxidized form, and so cannot be used as an oxygen delivery porter. Recently, interesting results about the carcinogenic effects of dietary haemin were reported, documenting that dietary haemin increases the number of aberrant crypt foci in rat colon mucosa[8-10]. It is important to understand how reactive oxygen species (ROS) are formed in the gut lumen and which biological potency they may have, since intracellular reactions with active oxygen can result in the initiation and progression of carcinogenesis by induction of gene mutations, chromosomal damage and cytotoxic effects[11-13]. Furthermore, active oxygen regulates expression of genes active during cell differentiation and growth and therefore, probably plays an important role in the promotion phase of tumor generation. In the colon, iron is expected to increase the production of ROS from peroxides via the Fenton reaction, which may be the cause of cellular toxicity and even pro-mutagenic lesions[14].

The aim of this study was to investigate whether hemoglobin could be classified as a proliferative agent for colon cancer cells by causing reactive oxygen species

release. For this purpose, we have studied the cellular viability of differentiated colon cell line HT29 after administration of hemoglobin at different concentrations. ROS production was investigated in each step. Additionally, we examined the protective effect of hemoglobin on the cytotoxicity of chemotherapeutic agents.

MATERIALS AND METHODS

Reagents and antibodies

Human hemoglobin, 5-fluorouracil (5-FU), 5'-deoxy-5-fluorouridine (5-DFUR) were obtained from Sigma (St. Louis, MO, USA). 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Calbiochem (Meudon, France). Antibodies to human peroxiredoxin 1 and superoxide dismutase 1 were purchased from Labfrontier (Seoul, Korea).

Cell culture

The human colon cancer cell line HT-29 and Lovo was established from Korean Cell Line Bank. To compare the effect on normal fibroblast, we used CCD-33Co normal colonic fibroblast cell line purchased from American Type Culture Collection (Catalog No. CRL-1539). Cells were maintained in stocks of liquid nitrogen, thawed and grown in tissue culture flasks with RPMI 1640 (Gibco BRL, NY, USA) supplemented with 100 mL/L fetal bovine serum and 10 g/L penicillin/streptomycin at 37℃ in a 50 mL/L CO_2 incubator. The cultured cells were trypsinized with fresh 2.5 g/L trypsin solution, trypsin was removed and the culture let sit at 37℃ until the cells detached (about 5 min). Fresh media was added, aspirated and dispensed into new flasks. Subculture was done every 4-6 d.

MTT assay

A freshly prepared cell suspension was serially diluted in RPMI 1640 containing 100 mL/L FBS to give a cell density ranging 10^9/L to 10^{11}/L counted by hemocytometer. After 24 h, the culture medium was replaced with a fresh medium containing hemoglobin, 5-FU, 5-DFUR or combination thereof. Six duplicate wells were set up in each sample. The cells not treated with the drugs served as control cells. After incubation time passed, 20 mL dimethylthiazol diphenyl tetrazolium bromide (MTT, 3 g/L) was added to each well and incubated at 37℃ for 3 h. After removal of the medium, MTT stabilization solution (DMSO: ethanol = 1:1) was added, then shaken for 10 min until all crystal was dissolved. Then, optical density (OD) was detected in a microplate reader at 550 nm wavelength using an ELISA reader (EMAX ED927, Molecular Devices Inc., USA). The negative control well had no cells and was used as zero point of absorbance. Each assay was performed in triplicate. The following formula was used: cell proliferation inhibited (%) = [1-(*A* of the experimental samples/*A* of the control)] × 100%. Cell growth curve was completed using time as the abscissa and a value (mean ± SD) as the ordinate.

Measurement of ROS

Human hemoglobin was dissolved in RPMI 1640. HT 29 cells were incubated with 5-FU or 5'-fluoro-2'-deoxyuridine (5-FDUR) at 37℃ in suspension culture at different concentrations 1 to 2 μg or for different incubation periods. These samples were processed for analysis of ROS by usual flowcytometric techniques. Briefly, cells were harvested, washed twice with PBS and resuspended in serum-free medium. They were incubated with 50 μmol/L 2',7'-dichlorofluoroscein diacetate (Calbiochem, Meudon, France) for 2 h at 37℃ and washed with ice-cold HEPES/saline and placed on ice. Fluorescence was measured by flowcytometry (Becton Diskinson, San Jose, USA). As a positive control, cells were separately treated with H_2O_2 and processed for ROS detection.

Immunoblot analysis

Harvested cell line extracts were homogenized with ice-cold lysis buffer (20 mmol/L HEPES pH 7.2, 150 mmol/L sodium chloride, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mg/L leupeptin, 10 mg/L aprotinine, 0.1 mmol/L DTT and 1 mmol/L phenylmethylsulfonyl fluoride). For the immunoblot analysis, extracted proteins of 10 μg were denatured by heating at 95℃ for 10 min with Laemmli cooking buffer and separated on 120 g/L SDS polyacrylamide gel electrophoresis. The resolved protein bands were transferred onto a PVDF membrane (Amersham Biosciences, UK) and blocked non-specific binding site by immersing the membrane in 30 mL/L skim milk, 120 g/L Tween 20 in TBS for 2 h. The membrane was incubated with 1:2000 diluted primary antibody for peroxiredoxin I (Difco, USA) and superoxide dismutase 1 for 1 h at room temperature on an orbital shaker. The blotted membrane was then incubated using secondary antibody (anti-rabbit IgG, 1:3000) for 1 h at room temperature on an orbital shaker. Detection was performed with the ECL system.

Statistical analysis

Data shown in figures represent mean ± SEM. Unless otherwise stated, these means were calculated from the means of triplicate replicates obtained in at least three independent experiments. Statistical evaluation was performed with the Prism program version 6.0. Depending on sample size and type of experiment, repeated measures of ANOVA or one-way ANOVAs were used to determine the significance of the experimental variables.

RESULTS

Proliferation effect of hemoglobin

Cell growth was determined by MTT assay. HT-29 cell line, the main cell of colon cancer, and Lovo cell line and CCL-33Co cell line were objected to confirm the difference in proliferation dependant on the types of cells. It has been verified, as demonstrated in Figure 1, when injected with hemoglobin, proliferation in all three types of cell lines was much greater. We saw the time effects in HT-29 cell line. These effects began to display after 30 min of treatment and were most obvious after approximately 24 h (Figure 1A). After various adjustments of the concentration of the hemoglobin, the results showed that the higher

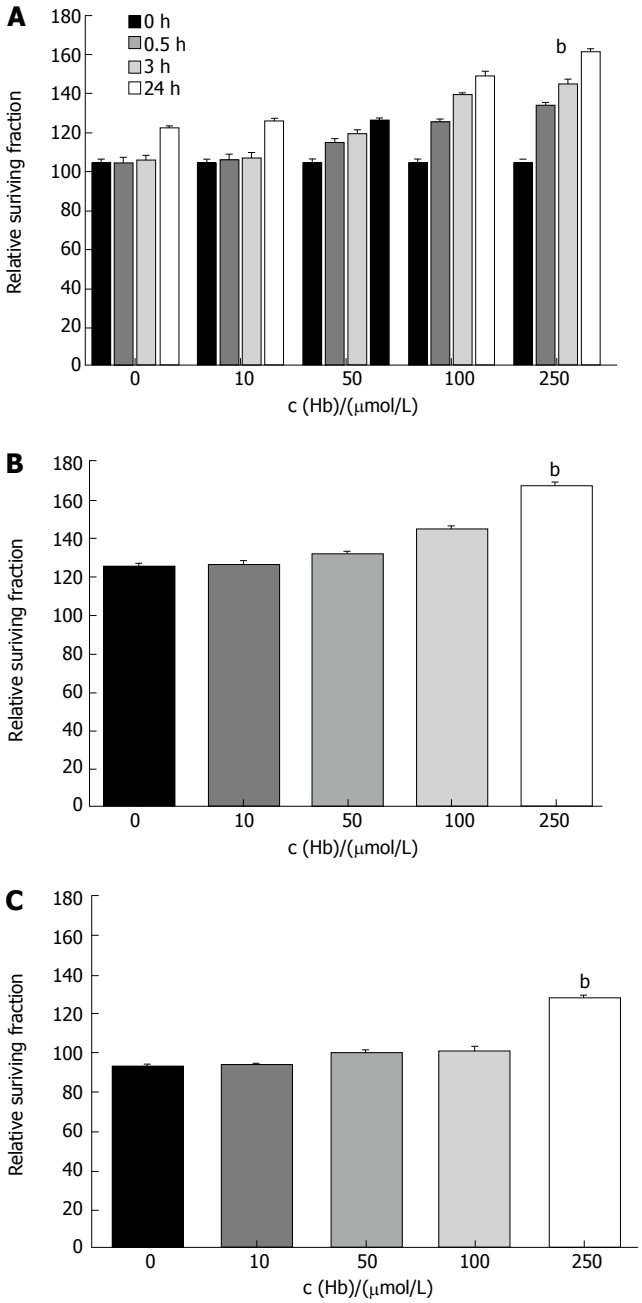


Figure 1 Time-course and dose-response effects of hemoglobin on apoptosis of colorectal cancer cell lines. **A**: HT-29 cell line. $^{b}P = 0.000$ *vs* 0 μmol/L; **B**: Lovo cell line. $^{b}P = 0.000$ *vs* 0 μmol/L; **C**: CCL-33Co cell line. $^{b}P = 0.000$ *vs* 0 μmol/L.

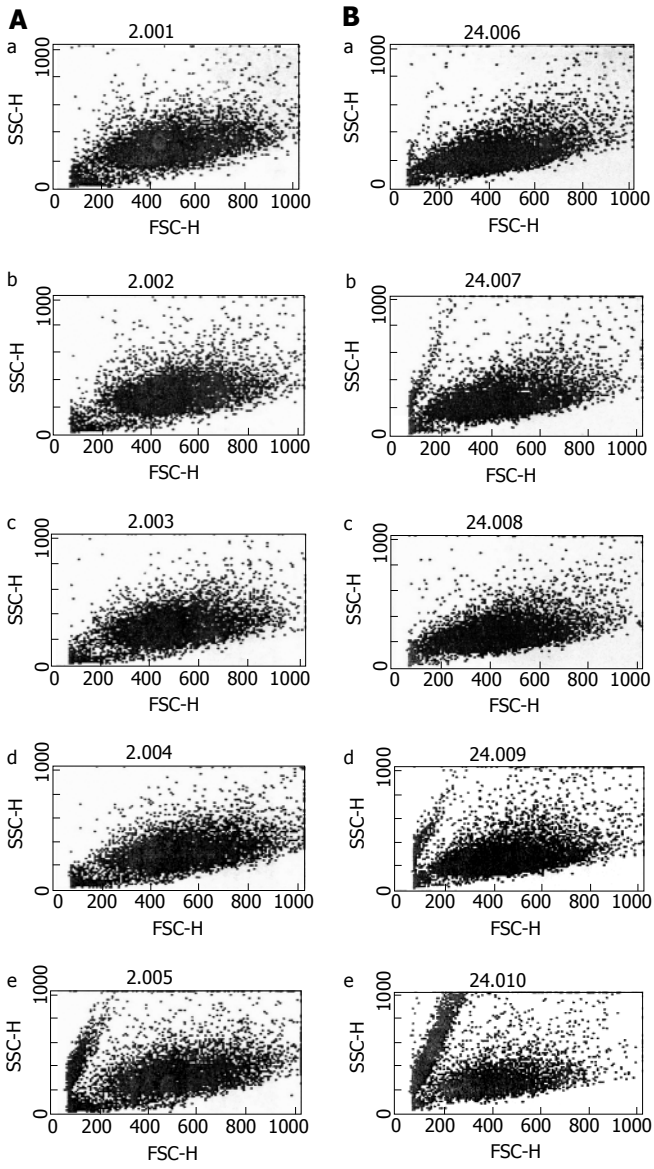


Figure 2 Flowcytometry of reactive oxygen species on HT-29 cells. **A**: After 3 h of hemoglobin administration; **B**: After 24 h of hemoglobin administration. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 50 μmol/L; d: Hb 100 μmol/L; e: Hb 250 μmol/L.

the concentration, the greater the proliferation (Figure 1).

ROS production by hemoglobin

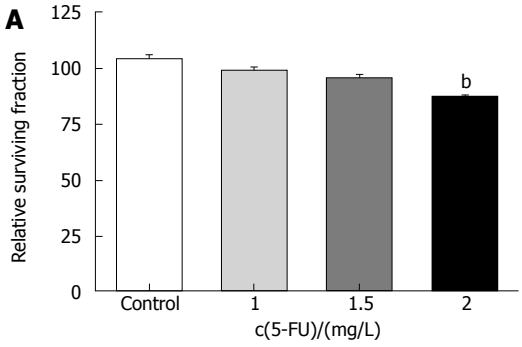
Flowcytometry using DEPC was conducted to measure the total quantity of ROS, which occurs during the administration of hemoglobin. As expected, the amount of ROS production showed a significant increase dependent on the concentration of hemoglobin (Figure 2), and ROS production increased subject to the amount of time following increment.

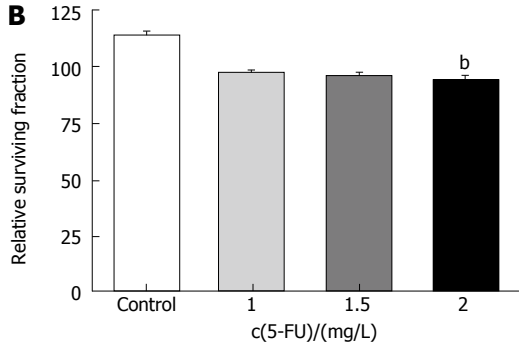
The restraint of proliferation by anti-cancer medicines

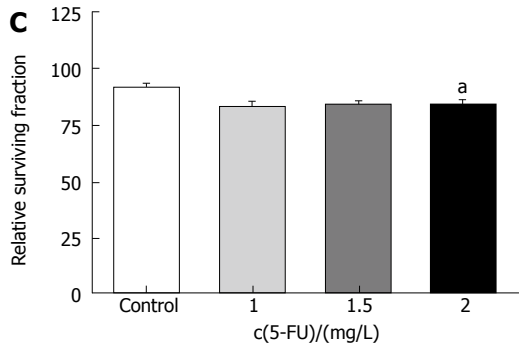
Two drugs (5-FU, an anti-cancer drug most commonly used in treatment of colon cancer, and 5-DFUR, the activating form of capecitabine which is presently used in metastatic colon cancer) were added to verify the constraint of proliferation effects. Twenty-four hours after drug application at varying concentrations, it was found that the higher the density, the greater was the constraint of proliferation in all three cell types (Figures 3 and 4). Also by flowcytometry, the anti-cancer medication decreases ROS production. Hence, it is believed that administration of anti-cancer drugs is effective in reducing ROS production (Figures 3D and 4D).

Influence of hemoglobin on anti-cancer agents

Twenty-four hours after simultaneously adding 5-FU, 5-DFUR and hemoglobin at various concentrations into three cell lines, results showed evidence of a weakening in the decreased proliferation as compared to using only anti-cancer drugs. Results were proportionate to the given densities of hemoglobin (Figures 5 and 6).







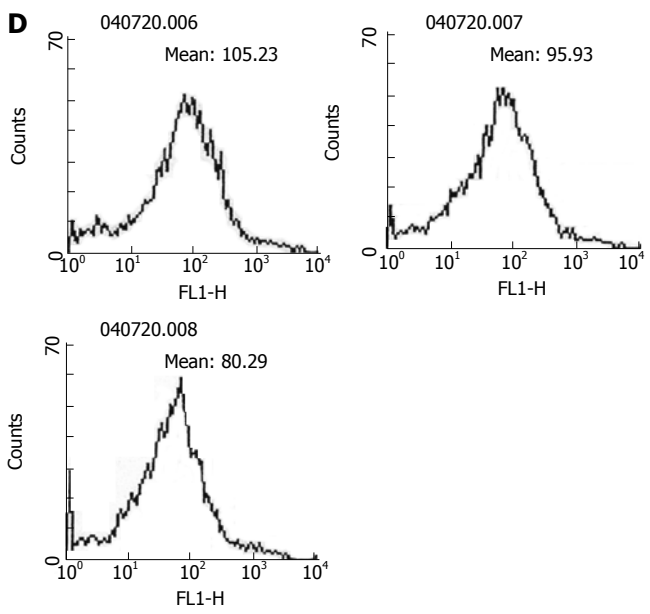
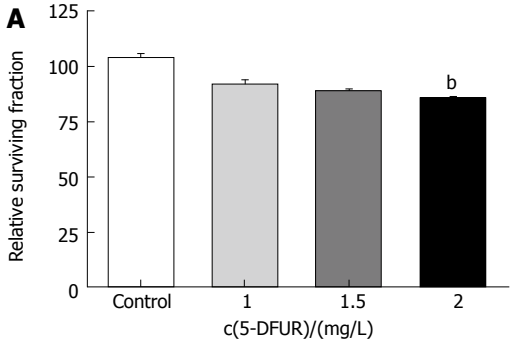
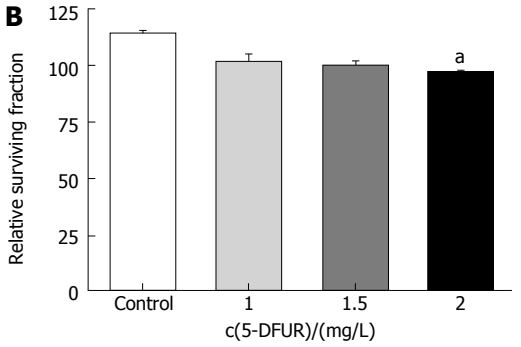
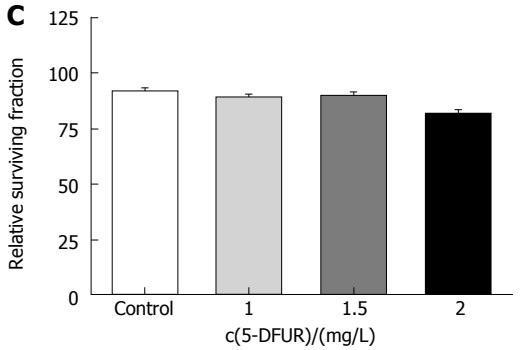


Figure 3 Relative surviving fraction of cell lines at 24 h after simultaneously adding each concentration of 5-fluorouracil. **A**: HT-29 cell line. [b]*P* = 0.001 *vs* Control; **B**: Lovo cell line. [b]*P* = 0.003 *vs* Control; **C**: CCL-33Co cell line. [a]*P* = 0.039 *vs* Control.







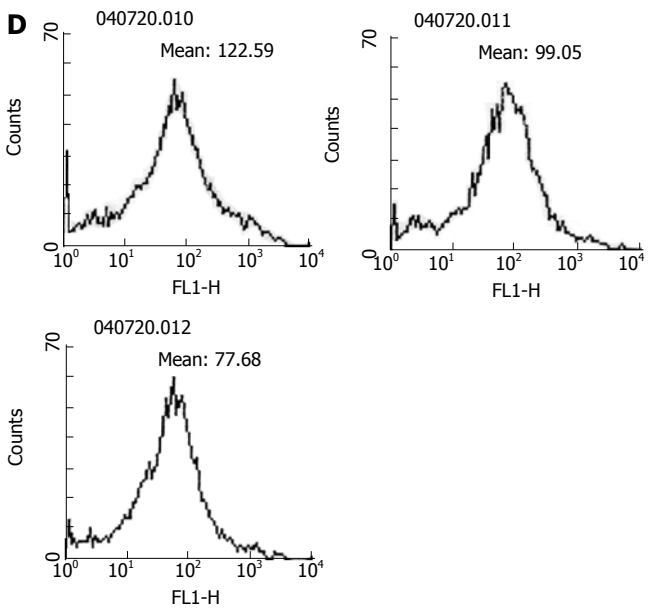


Figure 4 Relative surviving fraction of cell lines at 24 h after simultaneously adding each concentration of 5-DFUR. **A**: HT-29 cell line. [b]*P* = 0.001 *vs* Control; **B**: Lovo cell line. [b]*P* = 0.003 *vs* Control; **C**: CCL-33Co cell line. [b]*P* = 0.010 *vs* Control.

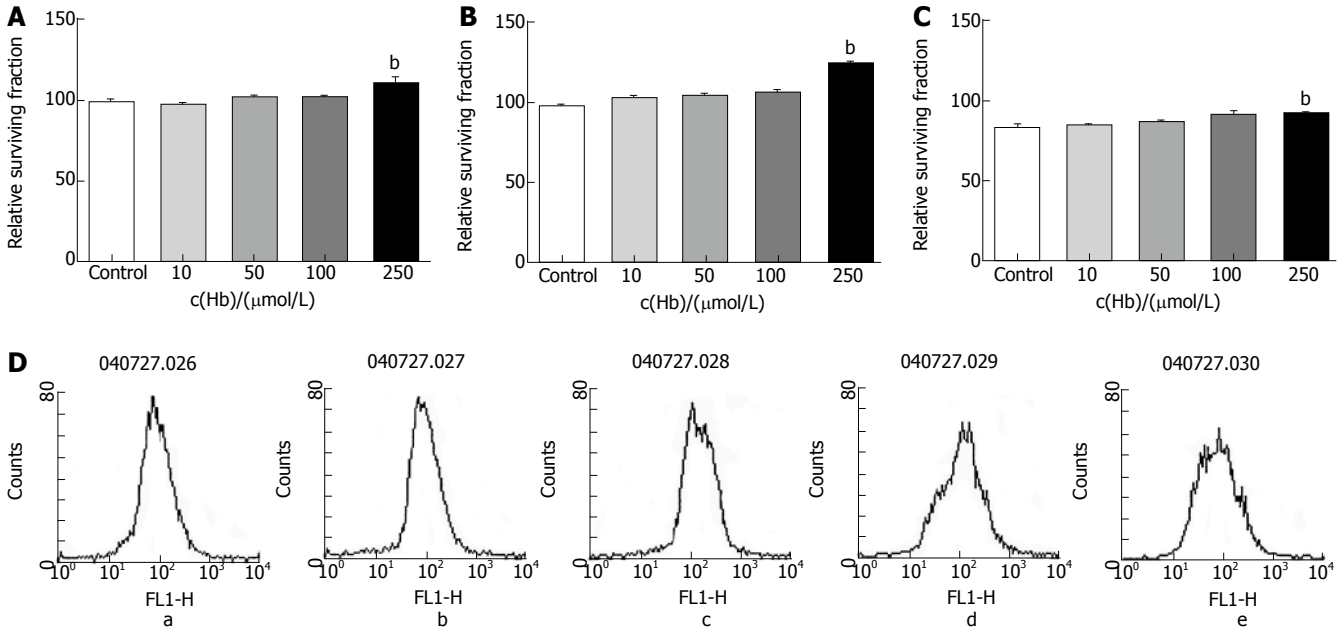


Figure 5 Relative surviving fraction of cell lines after adding 5-FU (1 mg/L) and each concentration of hemoglobin. **A**: HT-29 cell line. $^{b}P = 0.004$ *vs* Control; **B**: Lovo cell line. $^{b}P = 0.000$ *vs* Control; **C**: CCL-33Co cell line. $^{b}P = 0.005$ *vs* Control; **D**: Flowcytometry of HT-29 cell line. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 50 μmol/L; d: Hb 100 μmol/L; e: Hb 250 μmol/L.

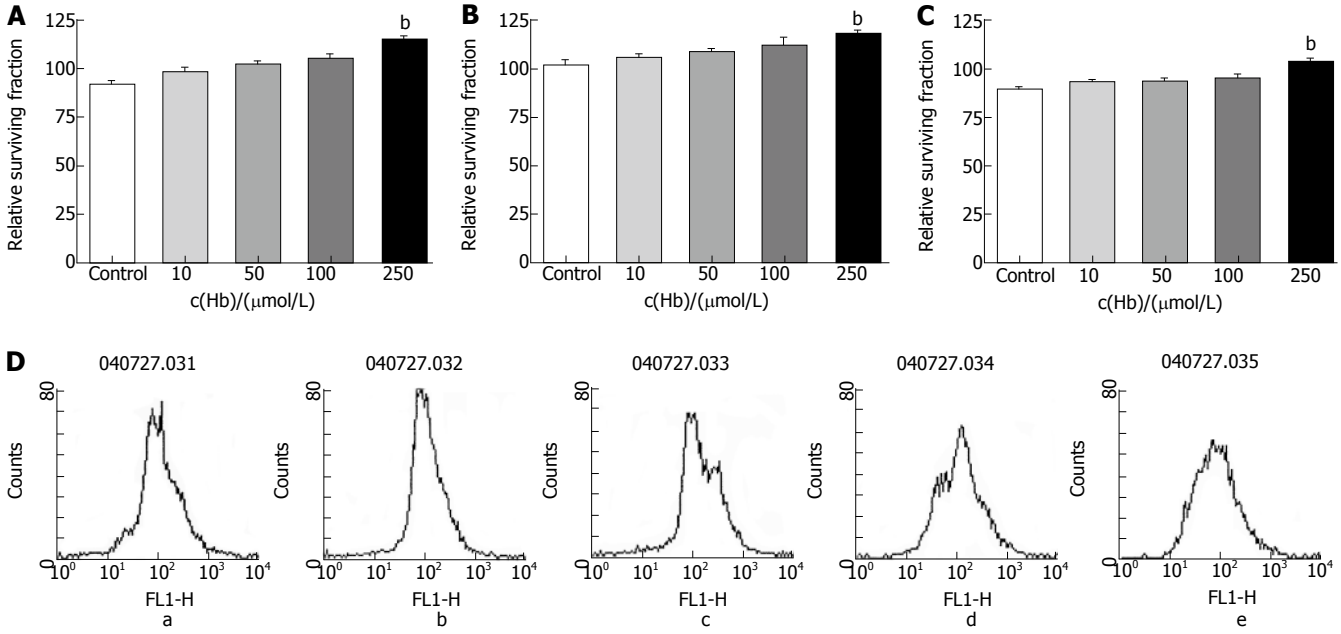


Figure 6 Relative surviving fraction of cell lines after adding 5-DFUR (1 mg/L) and each concentration of hemoglobin. **A**: HT-29 cell line. $^{b}P = 0.000$ *vs* Control; **B**: Lovo cell line. $^{b}P = 0.006$ *vs* Control; **C**: CCL-33Co cell line. $^{b}P = 0.000$ *vs* Control; **D**: Flowcytometry of HT-29. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 50 μmol/L; d: Hb 100 μmol/L; e: Hb 250 μmol/L.

Expression of superoxide dismutase and peroxiredoxin

The occurrence of superoxide dismutase, the most important rate-limiting enzyme in ROS production *in vivo*, and peroxiredoxin, which is a producer of hydrogen peroxide (prominent type of ROS in cancer cells), was compared by the amount of hemoglobin added. Twenty four hours following treatment, the rate of occurrence of the two types of enzymes increased proportionately to the density of hemoglobin. However, the variation was only slight and less than expected (Figures 7 and 8).

DISCUSSION

High intake of meat is believed to be one of the main factors of high incidence of colon cancer among Western countries as compared with Asian countries. There are many reports dealing with which components and nutrients inside meat are considered most risky. There are plenty of reports about nutrients such as iron[15], folate[16], cholesterol[17], calcium[18], bile salt[19], heterocyclic amines[20] and vitamin D[21] *etc.* related with various gastrointestinal malignancies. It is generally known that red meat has more

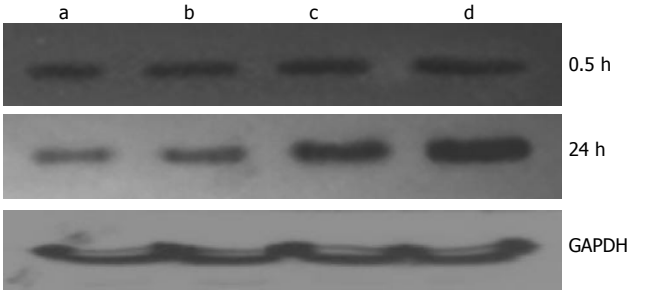


Figure 7 Change of Prx1 expression after administration of hemoglobin. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 100 μmol/L; d: Hb 250 μmol/L.

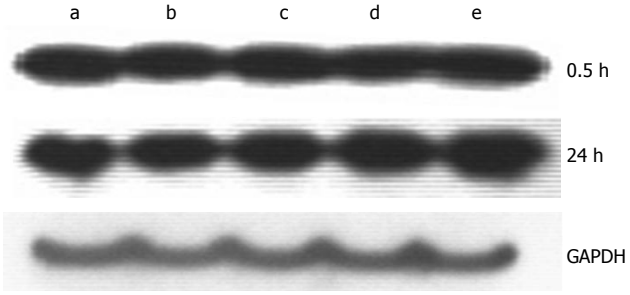


Figure 8 Change of SOD1 expression after administration of hemoglobin. a: Hb 0 μmol/L; b: Hb 10 μmol/L; c: Hb 50 μmol/L; d: Hb 100 μmol/L; e: Hb 250 μmol/L.

carcinogenic content than white meat. Due to the fact that some cancer patients believe red meat induces cancerous growths or encourages the recurrence thereof, they consume little to no red meats. Occasionally, malnutrition occurs as a result of extreme or reckless diet changes[22]. However, conclusions about carcinogenic effects of red meat are not confirmed to date, especially the medical basis is insufficient. Therefore, *in vitro* research is required to effectively prove these carcinogenic effects.

The term 'red meat' refers mainly to mammal flesh such as beef, pork and lamb, *etc*. as with most edible meats, whereas the term 'white meat' refers mainly to foul. The ruddy coloring of red meat reflects the density of myoglobin that is found in muscle tissue and some roles of hemoglobin are also included. Hemoglobin is a tetramer consisting of two alpha chains and two beta chains. Since each unit can combine with oxygen wherein iron is present, 1 hemoglobin can carry 4 oxygen molecules. The main function of hemoglobin is to carry oxygen, in addition to the storage of iron and the place providing globin. This type of function occurs only *in vivo* and exists within the bloodstream. When hemoglobin is inducted into the digestive tract, it is dissolved by digestive enzymes as are other proteins and absorbed as a protein and other nutrients. There are a few reports on the carcinogenic effects of heme or hemoglobin among the components of meat[10, 23-27]. Sesink *et al*[28] stated that dietary heme effects on colon epithelial hyperproliferation are hindered by calcium. They administered pure hemin in a meal of an F344 female rat. They announced that the resulting effect of a low calcium and heme containing diet increases colon wall aberrant crypt foci (ACF). Into an experimental model by Pierre *et al*[10] heme and hemoglobin were injected and the size and number of ACF were compared and measured with fecal thiobarbaturic acid reactive substances (TBAR). In the case of hemoglobin, the number of ACF and the amount of TBAR increased. From these results, hemoglobin was announced as a potent promoter of colorectal carcinogenesis. Glei *et al*[14] observed the increase of DNA strand break after the iron overload injected Fe-NTA (ferric-nitrilotriacetate) which was synthesized from the ferric nitrate and nitrilotriacetic acid into HT29 clone 19A cell line. Also, it was reported that when peroxide was added, more DNA strand breaks occurred with increasing peroxide concentrations. With these findings, iron content within a hemoglobin containing diet increased the DNA genotoxicity. We proceeded with this study under the assumption that hemoglobin components of dietary red meat affects cell proliferation of the cancerous and normal colonic cells via production of ROS. As expected, the results of this study confirmed the leading effect of hemoglobin. By the amount of hemoglobin dealt with, not only the cellular proliferation increased but also the amount of ROS production increased supporting predictions thereof. The proliferation effect of hemoglobin on normal colonic fibroblast was noted similarly in cancer cell lines. It is assumed that it presents the effect that exposure of hemoglobin leads to normal colon proliferation as well and produces ROS, which forms much more DNA breaks and also helps in becoming susceptible to other carcinogenic stimuli. Enzymes related with ROS production such as peroxiredoxin 1[29] and superoxide dismutase 1[30] also increased after hemoglobin application but the effect was minimal compared with expectations. These results suggested that production of enzymes was not a major factor in the process of colon cancer cell proliferation via ROS production.

5-FU and 5-DFUR are major chemotherapeutic agents in the treatment of colorectal cancer[31,32]. We used these drugs to identify adverse effects of hemoglobin on chemotherapy. Cellular proliferation was decreased 12 h after adding the 5-FU or 5-DFUR and ROS production was decreased as expected. The results with 5-FU was similar with that of 5-DFUR. These reactions were affected by administration of hemoglobin in a concentration-dependent manner in all three cell lines. ROS production was also decreased with hemoglobin. It is interpreted that hemoglobin administration restricted the cytotoxic effect of anti-cancer drugs in colon cancer cells and normal colonic fibroblasts.

In conclusion, hemoglobin inside red meat has a promoting effect on cellular proliferation in cancer cells and in normal colonic fibroblast cells by release of ROS. Furthermore, this phenomenon reduces the cytotoxicity of anticancer drugs, such as 5-FU and 5-DFUR, to colon cancer cells, which could be an adverse factor during chemotherapy in a clinical setting.

REFERENCES

1 **Ransohoff DF.** Colon cancer screening in 2005: status and challenges. *Gastroenterology* 2005; **128**: 1685-1695
2 **Ministry of health and welfare of Korea.** Reports of cancer incidence 07-27. Available from: http://www.mohw.go.kr/in-

dex.jsp
3 **Li M,** Gu J. Changing patterns of colorectal cancer in China over a period of 20 years. *World J Gastroenterol* 2005; **11**: 4685-4688
4 **Ferguson LR.** Meat consumption, cancer risk and population groups within New Zealand. *Mutat Res* 2002; **506-507**: 215-224
5 **Matos E,** Brandani A. Review on meat consumption and cancer in South America. *Mutat Res* 2002; **506-507**: 243-249
6 **Nkondjock A,** Ghadirian P. Associated nutritional risk of breast and colon cancers: a population-based case control study in Montreal, Canada. *Cancer Lett* 2005; **223**: 85-91
7 **Young GP,** Rose IS, St John DJ. Haem in the gut. I. Fate of haemoproteins and the absorption of haem. *J Gastroenterol Hepatol* 1989; **4**: 537-545
8 **Shivshankar P,** Devi SC. Screening of stimulatory effects of dietary risk factors on mouse intestinal cell kinetics. *World J Gastroenterol* 2005; **11**: 242-248
9 **Kim DH,** Hong EK. Aberrant crypt foci in the background mucosa of colorectal adenocarcinoma. *Cancer Res Treatment* 2001; **33**: 216-224
10 **Pierre F,** Taché S, Petit CR, Van der Meer R, Corpet DE. Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats. *Carcinogenesis* 2003; **24**: 1683-1690
11 **Kamata H,** Hirata H. Redox regulation of cellular signalling. *Cell Signal* 1999; **11**: 1-14
12 **Forsberg L,** de Faire U, Morgenstern R. Oxidative stress, human genetic variation, and disease. *Arch Biochem Biophys* 2001; **389**: 84-93
13 **Benhar M,** Engelberg D, Levitzki A. ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep* 2002; **3**: 420-425
14 **Glei M,** Latunde-Dada GO, Klinder A, Becker TW, Hermann U, Voigt K, Pool-Zobel BL. Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A. *Mutat Res* 2002; **519**: 151-161
15 **Lee DH,** Jacobs Jr DR, Folsom AR. A hypothesis: interaction between supplemental iron intake and fermentation affecting the risk of colon cancer. The Iowa Women's Health Study. *Nutr Cancer* 2004; **48**: 1-5
16 **Kim YI.** Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility. *J Nutr* 2005; **135**: 2703-2709
17 **Suzuki K,** Ito Y, Wakai K, Kawado M, Hashimoto S, Toyoshima H, Kojima M, Tokudome S, Hayakawa N, Watanabe Y, Tamakoshi K, Suzuki S, Ozasa K, Tamakoshi A. Serum oxidized low-density lipoprotein levels and risk of colorectal cancer: a case-control study nested in the Japan Collaborative Cohort Study. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 1781-1787
18 **Gross MD.** Vitamin D and calcium in the prevention of prostate and colon cancer: new approaches for the identification of needs. *J Nutr* 2005; **135**: 326-331
19 **Alberts DS,** Martínez ME, Hess LM, Einspahr JG, Green SB, Bhattacharyya AK, Guillen J, Krutzsch M, Batta AK, Salen G, Fales L, Koonce K, Parish D, Clouser M, Roe D, Lance P. Phase III trial of ursodeoxycholic acid to prevent colorectal adenoma recurrence. *J Natl Cancer Inst* 2005; **97**: 846-853
20 **Pence BC,** Landers M, Dunn DM, Shen CL, Miller MF. Feeding of a well-cooked beef diet containing a high heterocyclic amine content enhances colon and stomach carcinogenesis in 1, 2-dimethylhydrazine-treated rats. *Nutr Cancer* 1998; **30**: 220-226
21 **Harris DM,** Go VL. Vitamin D and colon carcinogenesis. *J Nutr* 2004; **134**: 3463S-3471S
22 **Lee KS,** Ahn HS, Hwang LI, Lee YS, Koo BH. Utilization of alternative therapies in cancer patients. *J Korean Cancer Assoc* 1998; **30**: 203-213
23 **Glei M,** Klenow S, Sauer J, Wegewitz U, Richter K, Pool-Zobel BL. Hemoglobin and hemin induce DNA damage in human colon tumor cells HT29 clone 19A and in primary human colonocytes. *Mutat Res* 2006; **594**: 162-171
24 **Lee DH,** Anderson KE, Harnack LJ, Folsom AR, Jacobs DR Jr. Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health Study. *J Natl Cancer Inst* 2004; **96**: 403-407
25 **Pierre F,** Freeman A, Taché S, Van der Meer R, Corpet DE. Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons. *J Nutr* 2004; **134**: 2711-2716
26 **Lakshmi VM,** Clapper ML, Chang WC, Zenser TV. Hemin potentiates nitric oxide-mediated nitrosation of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) to 2-nitrosoamino-3-methylimidazo[4,5-f]quinoline. *Chem Res Toxicol* 2005; **18**: 528-535
27 **Bingham SA,** Hughes R, Cross AJ. Effect of white versus red meat on endogenous N-nitrosation in the human colon and further evidence of a dose response. *J Nutr* 2002; **132**: 3522S-3525S
28 **Sesink AL,** Termont DS, Kleibeuker JH, Van der Meer R. Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium. *Carcinogenesis* 2001; **22**: 1653-1659
29 **Noh DY,** Ahn SJ, Lee RA, Kim SW, Park IA, Chae HZ. Overexpression of peroxiredoxin in human breast cancer. *Anticancer Res* 2001; **21**: 2085-2090
30 **St Clair D,** Zhao Y, Chaiswing L, Oberley T. Modulation of skin tumorigenesis by SOD. *Biomed Pharmacother* 2005; **59**: 209-214
31 **Reddy GK.** Efficacy of adjuvant capecitabine compared with bolus 5-fluorouracil/leucovorin regimen in dukes C colon cancer: results from the X-ACT trial. *Clin Colorectal Cancer* 2004; **4**: 87-88
32 **Kim IY.** Improving outcomes with chemotherapy in colorectal cancer: current options, current evidence. *J Korean Soc Coloproctol* 2006; **22**: 137-148

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Promoter hypomethylation and reactivation of *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines and cancer tissues

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Abstract

AIM: To verify the expression and methylation status of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues and cancer cell lines.

METHODS: We evaluated promoter demethylation status of the *MAGE-A1* and *MAGE-A3* genes by RT-PCR analysis and methylation-specific PCR (MS-PCR), as well as sequencing analysis, after sodium bisulfite modification in 32 colorectal cancer cell lines and 87 cancer tissues.

RESULTS: Of the 32 cell lines, *MAGE-A1* and *MAGE-A3* expressions were observed in 59% and 66%, respectively. Subsequent to sodium bisulfite modification and MS-PCR analysis, the promoter hypomethylation of *MAGE-A1* and *MAGE-A3* was confirmed in both at 81% each. Promoter hypomethylation of *MAGE-A1* and *MAGE-A3* in colorectal cancer tissues was observed in 43% and 77%, respectively. Hypomethylation of *MAGE-A1* and *MAGE-A3* genes in corresponding normal tissues were observed in 2% and 6%, respectively.

CONCLUSION: The promoter hypomethylation of *MAGE* genes up-regulates its expression in colorectal carcinomas as well as in gastric cancers and might play a significant role in the development and progression of human colorectal carcinomas.

Key words: *MAGE-A1*; *MAGE-A3*; Promoter; Hypomethylation; Colorectal cancer

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INTRODUCTION

Human tumors often display changes in DNA methylation, which include both genome-wide hypomethylation and site-specific hypermethylation. Global hypomethylation and CpG island hypermethylation have been recognized as important contributors to the development of carcinogenesis in humans. Hypermethylation of promoter CpG islands is the signature of transcriptional silencing of their downstream genes, including *RB, p16, VHL, BRCA1, E-cadherin, APC, hMLH1, FHIT, COX2,* and *CDX1* in various human cancers; and is as effective as inactivation by gene mutation or deletion[1-6]. Global DNA hypomethylation has been implicated in the activation of oncogenes such as *c-myc, k-ras*, and it may also contribute to tumor progression by the induction of genome instability[7,8].

The *MAGE* family of genes belongs to a group of germ line-specific genes that are activated in different types of tumors. This family of genes was reported to direct the expression of a tumor-specific antigen that was recognized in a melanoma cell by cytolytic T lymphocytes[9]. The *MAGE-A1* gene has a CpG-rich promoter, which, unlike classical CpG-rich promoters, is methylated in all normal somatic tissues, except for the placenta and testis. In contrast, the promoter region of *MAGE-A1* is completely unmethylated in testicular germ cells and in tumor cells that express the gene[10]. Demethylation, and therefore, activation of *MAGE-A1* in tumors appears to be a consequence of the genome-wide demethylation process, since the expression of this gene in tumor cells correlates with a decreased level of overall DNA methylation[11]. A correlation between *MAGE-A1* and *MAGE-A3* expression and genome-wide hypomethylation has been observed in some types of carcinomas[12,13]. The human *MAGE-A1* and *MAGE-A3* genes, which are located on chromosome X, are expressed in 29% and 66%, respectively, of human gastric cancer cells due to the hypomethylation of the promoter region[12]. However, it is unknown if this relationship is present in colorectal carcinomas.

In this study, we investigated the promoter methylation

status of *MAGE-A1* and *MAGE-A3* genes. A total of 32 colorectal cancer cell lines were tested for hypomethylation of the *MAGE-A1* and *MAGE-A3* genes promoter. In addition, we screened the methylation status of the *MAGE-A1* and *MAGE-A3* genes promoter in 87 paired colorectal cancers and normal mucosal tissue samples.

MATERIALS AND METHODS

Cell cultures

A total of 32 colorectal cancer cell lines (Table 1) and 2 gastric cancer cell lines (SNU-1 and SNU-5) were obtained from either the Korean Cell Line Bank (KCLB; Seoul, Korea) or the American Type Culture Collection (ATCC; Manassas, VA, USA). Sixteen SNU-colorectal cancer cell lines were established and were reported upon previously by this laboratory[14,15]. SNU-1 and SNU-5 gastric carcinoma cell lines were used as methylation positive (SNU-1) and negative (SNU-5) controls for *MAGE* gene expression[12]. All the cell lines were maintained in RPMI1640, which was supplemented with 10% FBS, 100 kU/L penicillin, and 0.1 g/L streptomycin. The cultures were maintained in humidified incubators at 37℃ in a 5% CO_2 and 95% ambient air atmosphere.

Nucleic acid isolation and cDNA synthesis from the cell lines

Genomic DNA and total RNA were isolated from washed-cell pellets. Total genomic DNA was extracted in accordance with the standard SDS-proteinase K procedure; and total cellular RNA was extracted based on the manufacturer's instructions (Intron Biotechnology; Seoul, Korea). For cDNA synthesis, 2 μg of total RNA was reverse transcribed with a random hexamer, dNTPs, and 1 μL (200 U) of Superscript™ II reverse transcriptase (Life Technologies; Gaithersburg, MD, USA) in a final volume of 20 μL for 1 h and 15 min at 42℃ after a 10-min denaturation at 70℃. Eighty microliters of distilled water were added subsequent to the reverse-transcription reaction.

Expression of MAGE-A1 and MAGE-A3 genes

For mRNA expression analysis, the cDNA was amplified in 25 μL of a PCR reaction mix with 1 μL of the reverse-transcription reaction, the primers and 0.5 U of Taq DNA polymerase. The PCR conditions consisted of 10 min at 94℃ for the initial denaturation, followed by 35 cycles of 94℃ for 30 s, 54℃ for 60 s, and 72℃ for 60 s, and a final elongation of 7 min at 72℃. The primer sequences are as follows. *MAGE-A1* cDNA was amplified by PCR with MG1 RT primers; MG1 RT sense, 5'-TGTGG GCAGGAGCTGGGCAA-3', MG1 RT antisense, 5'-GCCGAAGGAACCTGACCCAG -3'. For the *MAGE-A3* cDNA, the MG3 RT primers were used; MG3 RT sense, 5'-AAGCCGGCCCAGGCTCGGT-3', MG3 RT antisense, 5'-GCTGGGCAATGGAGACCCAC-3'. PCR amplification was performed in a programmable thermal cycler (PCR System 9700, Applied Biosystems; Foster City, CA, USA). Primers for *β-actin* were used to confirm RNA integrity. Both *MAGE-A1* and *MAGE-A3* and *β-actin* RT-PCR reactions used the same cDNA synthesis. The amplified DNA fragments were fractionated in 2% agarose gel and stained with ethidium bromide.

Tissue sample collection and DNA extraction

A total of 87 paired tumor and normal mucosal tissue samples were obtained from 87 patients, who had primary colorectal adenocarcinoma. The normal mucosal tissue specimens were collected from each patient 10 cm or more away from the tumor areas. Approximately 2 g of the surgically removed tissues were frozen immediately and then stored in liquid nitrogen. The remaining sections of the samples were fixed with formalin and used for further histological examination in order to confirm the diagnosis postoperatively. Genomic DNA was isolated from the frozen-tissue biopsies with the standard SDS-proteinase K procedure.

Methylation specific PCR

With respect to the MS-PCR, the sodium bisulfite modification of genomic DNA was performed as reported previously[16]. A total of 2 μg of genomic DNA obtained from colorectal cancer cell lines, was denatured with NaOH and hydroquinone. Then, 3 mmol/L sodium bisulfite was added and the mixture was incubated at 55℃ for 16 h. Following the bisulfite modification, the DNA was purified with a Wizard DNA purification system (Promega; Madison, WI, USA), ethanol precipitated, dried, and resuspended in 100 μL distilled water. The PCR was performed using the PCR primers that were described previously[12]. The amplified DNA fragments were fractionated in 2% agarose gel that was stained with ethidium bromide and visualized under UV light.

5-aza-2'-deoxycytidine treatment and RT-PCR

For 5-aza-2'-deoxycytidine treatment, the cells were seeded in two 2×10^5 cells/75 cm^2 culture flasks on d 0. The cells were treated with and without 1-5 μmol/L of 5-aza-2' -deoxycytidine (Sigma Chemical Co.) for 24 h on d 2 and 5, and the medium was changed 24 h after addition of 5-aza-2'-deoxycytidine. The cells were harvested on d 8 for the analysis of the *MAGE*s expression. Subsequently, the RNA was prepared, and RT-PCR was performed to detect *MAGE-A1* and *MAGE-A3* expression with the *MAGE-A1* and *MAGE-A3* RT-PCR primers as described above.

RESULTS

Expression of MAGE-A1 and MAGE–A3 in colorectal cancer cell lines

Expression of *MAGE-A1* and *MAGE-A3* mRNA in 32 colorectal cancer cell lines was analyzed by RT-PCR, and *MAGE-A1* and *MAGE-A3* expressions were observed in 19 (59%) and 21 (66%) of the cell lines, respectively (Figure 1 and Table 1). PCR for *β-actin* confirmed the integrity of the RNA.

Clinico-pathological features

Of the 87 colorectal carcinomas, 57 (66%) were obtained from the proximal colon (cecum to splenic flexure), and 30 (34%) from the distal colorectum (splenic flexure to

Table 1 Methylation status of the promoter region of *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines

	Cell line	*MAGEs* expression				MS-PCR			
		MAGE-A1		*MAGE-A3*		*MAGE-A1*		*MAGE-A3*	
		-5-aza/+5-aza		- 5-aza/+5-aza		M/U		M/U	
1	SNU-61	-	++	±	++	+	+	-	+
2	SNU-81	-	++	-	+++	+	-	+	-
3	SNU-175	++	++	+++	+++	+	+	+	+
4	SNU-283	+++	NT	+++	NT	+	+	+	-
5	SNU-407	+++	+	+++	++	+	-	+	+
6	SNU-503	++	NT	-	NT	+	+	+	+
7	SNU-769A	+++	++	+++	+++	-	+	-	+
8	SNU-769B	+++	+++	+++	+++	-	+	-	+
9	SNU-1033	++	+	++	+++	+	+	+	+
10	SNU-1040	-	-	±	++	+	-	+	-
11	SNU-1047	+++	++	++	++	+	-	-	+
12	SNU-1197	++	+++	++	+++	+	+	+	+
13	SNU-C1	±	NT	++	NT	+	+	-	+
14	SNU-C2A	++	NT	+++	NT	+	+	+	+
15	SNU-C4	-	NT	-	NT	+	-	+	+
16	SNU-C5	±	++	-	+++	+	+	+	-
17	Caco-2	-	++	++	+++	+	+	+	+
18	COLO201	-	++	-	+++	-	+	-	+
19	COLO205	-	-	-	++	+	+	+	+
20	COLO320	+++	NT	+++	NT	+	+	-	+
21	DLD-1	-	+++	-	+++	+	+	+	+
22	HCT-8	-	++	-	+++	+	-	+	-
23	HCT-15	-	++	-	+++	+	+	+	+
24	HCT-116	+++	NT	+++	NT	+	+	+	+
25	HT-29	+++	++	+++	+++	+	+	+	+
26	Lovo	+++	+++	+++	+++	+	+	+	+
27	LS174T	+++	NT	+++	NT	+	+	-	+
28	NCI-H716	+++	+++	+++	+++	+	+	-	+
29	SW403	+++	++	+++	+++	+	+	+	+
30	SW480	+++	NT	+++	NT	+	+	+	-
31	SW1116	-	NT	++	NT	+	+	-	+
32	WiDR	++	+++	+++	+++	+	+	-	+

5-aza: 5-aza-2′-deoxycytidine; NT: Not tested; M: The amplified product with primers recognizing methylated sequence; U: The amplified product with primers recognizing unmethylated sequence; +: Amplified product; -: Not amplified product.

rectum). Randomly selected patients aged 16-81 years, including 55 males and 32 females. Of the 32 colorectal cancer cell lines, 7 originated from the proximal colon and 8 from the distal colorectum. The origin of the remaining 17 colorectal cancer cell lines was unknown.

Analysis of MAGE-A1 and MAGE-A3 methylation by MS-PCR

By using primers for unmethylated *MAGE-A1* DNA amplification on bisulfite modified DNA, amplified DNA fragments were observed in 26 (81%) cell lines (SNU-61, SNU-175, SNU-283, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, SNU-C5, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW480, SW1116, and WiDR) (Figure 2 and Table 1). And by using primers for unmethylated *MAGE-A3* DNA amplification on bisulfite modified DNA, amplified DNA fragments were found also in 26 (81%) cell lines (SNU-61, SNU-175, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1047, SNU-1197A, SNU-C1, SNU-C2A, SNU-C4, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR) (Figure 2 and Table 1). By using primers for amplification of unmethylated or methylated DNA, amplified DNA fragments were found in all 32 cell lines. *MAGE-A1* unmethylated DNA products were observed in 37 out of 87 tumor tissue samples (43%; Figure 3). In the normal tissue samples, the methylated DNA was amplified in all 87 samples. However, the unmethylated DNA was amplified in 2 normal tissues (2%). *MAGE-A3* unmethylated DNA products were observed in 67 out of 87 tumor tissue samples (77%; Figure 3). In the normal tissue samples, the methylated DNA was amplified in all 87 samples. However, the unmethylated DNA was amplified in 5 normal tissues (6%).

Reexpression of MAGE-A1 and MAGE-A3 after treatment with 5-aza-2′-deoxycytidine

We investigated whether *MAGE-A1* mRNA was re-expressed after 5-aza-2'-deoxycytidine treatment in 22 cell

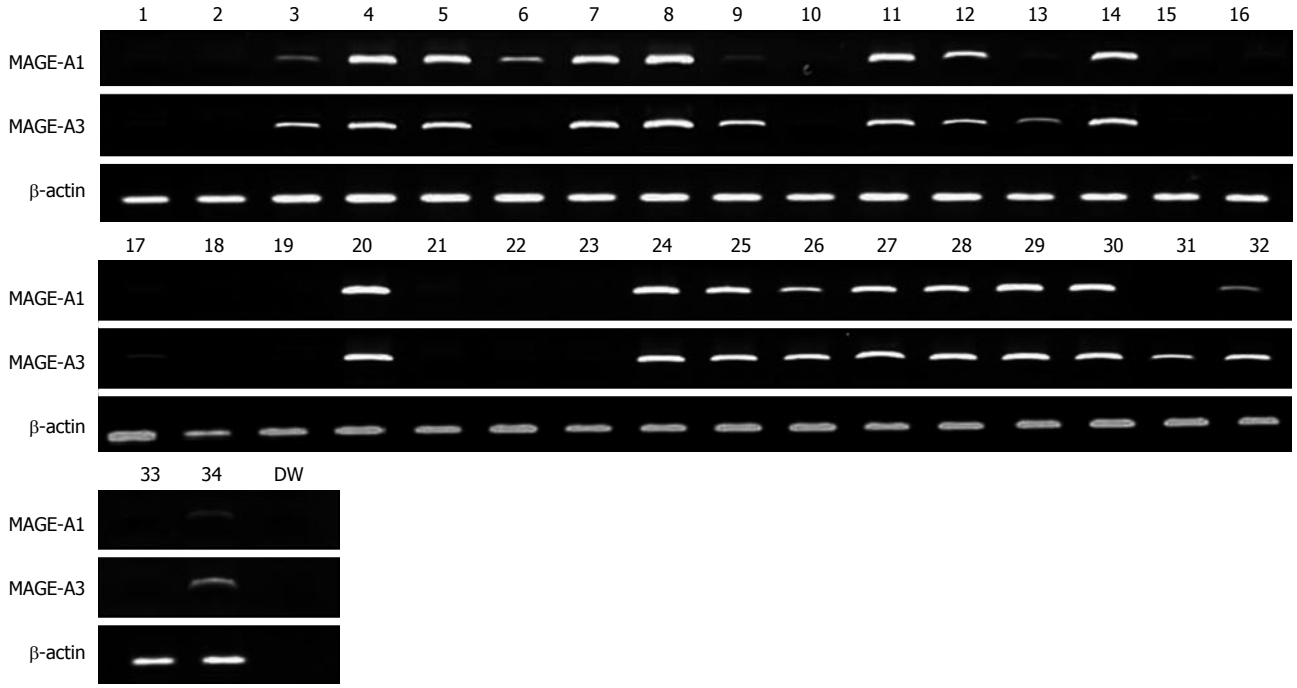

Figure 1 RT-PCR analysis of the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines. β-actin was amplified as an internal control. The *MAGE-A1* gene was significantly expressed in 19 colorectal cancer cell lines (Lanes, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 20, 24, 25, 26, 27, 28, 29, 30 and 32). The *MAGE-A3* gene was expressed in 21 colorectal cancer cell lines (Lanes, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14, 17, 20, 24, 25, 26, 27, 28, 29, 30, 31 and 32). Lane numbers 1 to 34 show cell lines SNU-61, SNU-81, SNU-175, SNU-283, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1040, SNU-1047, SNU-1197, SNU-C1, SNU-C2A, SNU-C4, SNU-C5, Caco-2, COLO201, COLO205, COLO320, DLD1, HCT-8, HCT-15, HCT-116, HT-29, LOVO, LS174T, NCI-H716, SW403, SW480, SW1116, WiDr, SNU-1, and SNU-5 respectively.

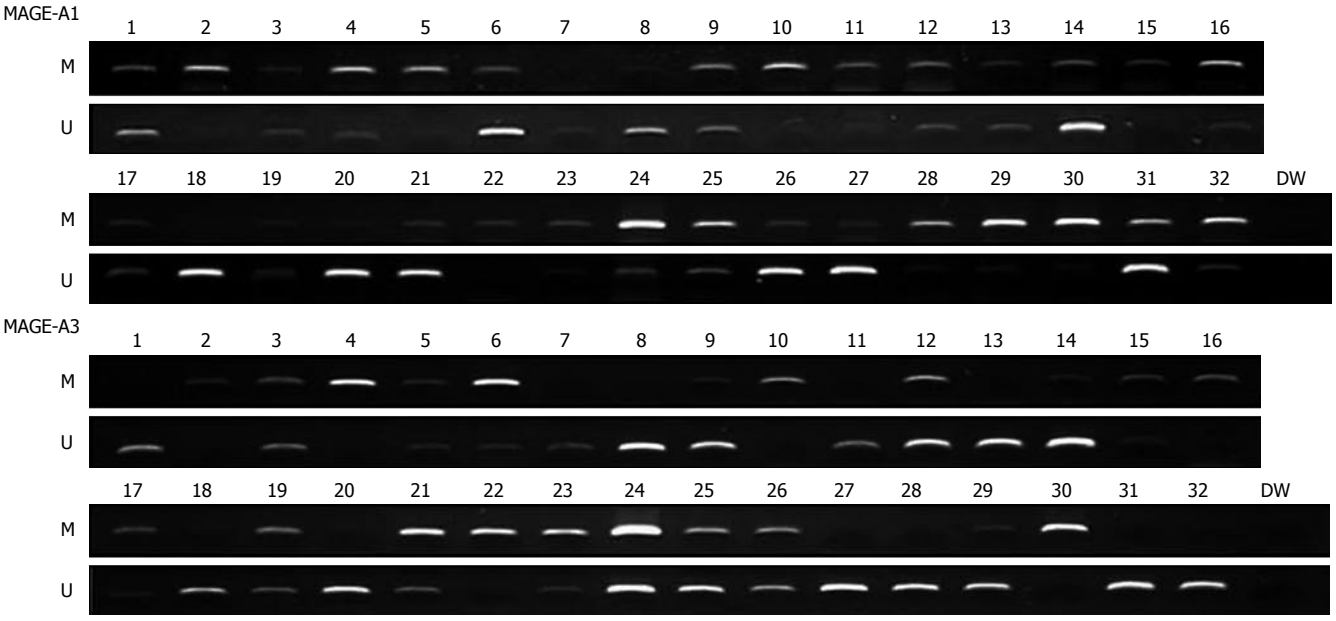

Figure 2 Methylation analysis of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines. The promoter region of the *MAGE-A1* gene was unmethylated in 26 cell lines (SNU-61, SNU-175, SNU-283, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, SNU-C5, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR cell lines). Unmethylated *MAGE-A3* DNA amplifications were found in 26 cell lines (SNU-61, SNU-175, SNU-407, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1047, SNU-1197A, SNU-C1, SNU-C2A, SNU-C4, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR cell lines). Lane M denotes product amplified by primers recognizing a methylated sequence and Lane U denotes the product amplified by primers recognizing an unmethylated sequence, respectively.

lines, including 10 cell lines (SNU-61, SNU-81, SNU-1040, SNU-C5, Caco-2, COLO201, COLO205, DLD1, HCT-8, and HCT-15) that did not express *MAGE-A1* mRNA. After an RT-PCR analysis, we observed that all of the *MAGE-A1* mRNAs were re-expressed, except for the SNU-1040 and COLO205 cell lines (Table 1). This suggested that the inactivation of *MAGE-A1* expression was caused by another mechanism. Further, we investigated whether *MAGE-A3* mRNA was re-expressed after 5-aza-2'-deoxycytidine treatment in 22 cell lines, including 9 cell lines (SNU-61, SNU-81, SNU-1040, SNU-C5, COLO201, COLO205, DLD1, HCT-8, and

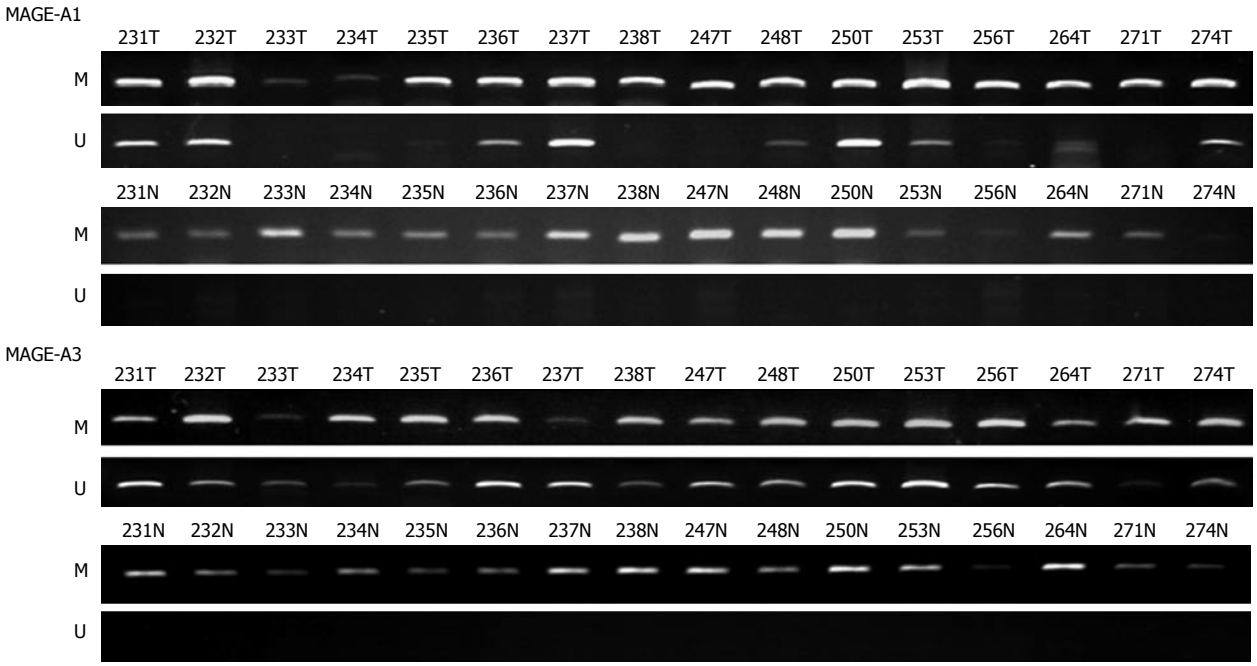

Figure 3 Methylation analysis of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues and corresponding normal tissues. Methylation-specific PCR product amplified by primers recognizing methylated and unmethylated sequence. The promoter region of the *MAGE-A1* and *MAGE-A3* genes was unmethylated in colorectal cancer tissues, not normal tissues. Numbers represent each colorectal tissue and T denotes colorectal tumor tissues and N denotes corresponding normal tissues.

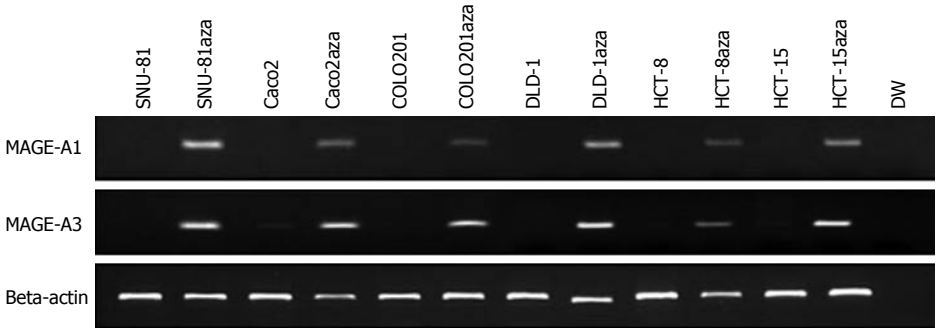

Figure 4 RT-PCR analysis after treatment with 5-aza-2'-deoxycytidine. The *MAGE-A1* and *MAGE-A3* genes were reactivated.

HCT-15) that did not express *MAGE-A3* mRNA. After an RT-PCR analysis, we observed that all of the *MAGE-A3* mRNAs were re-expressed (Table 1 and Figure 4).

DISCUSSION

Genome-wide hypomethylation and site-specific hypermethylation are common features of cancer cells. DNA hypomethylation in cancer cells is accompanied by the activation of germ line-specific genes, such as the *MAGE-A1* gene, the repression of which, in normal somatic tissues, is dependent upon DNA methylation[17]. Recent studies have reported the presence of very high *MAGE-A1* and *MAGE-A3* expressions in colorectal carcinomas[18,19]. Although previous reports have shown the expression of *MAGE* genes, the mechanism of *MAGE* genes expression in colorectal carcinomas was unclear. This led us to question whether it could be associated with decreased genomic methylation. It has been reported that *MAGE-A1* and *MAGE-A3* expression was related to gene hypomethylation in gastric carcinoma, hepatocarcinoma, and melanoma[12,20,21]. However, such a relationship has still not been confirmed in colorectal carcinoma. Accordingly, we analyzed the methylation status of the promoter region on the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines and estimated its association with *MAGE-A1* and *MAGE-A3* mRNA expression. We first examined the expression pattern of the *MAGE-A1* and *MAGE-A3* genes in these cell lines by an RT-PCR and observed that *MAGE-A1* and *MAGE-A3* were over-expressed significantly in 19 (59%) and 21 (66%) cell lines, respectively. This expression ratio of *MAGE-A1* and *MAGE-A3*, obtained by an RT-PCR in colorectal cancer cell lines, is similar to that observed in gastric cancer cell lines[12]. On the other hand, previous reports have revealed a much lower expression of MAGE in colorectal carcinomas ranging between 5%-39%[18,19,22]. In the literature, the expression of the *MAGE* genes was studied in colorectal carcinoma tissues; however, we have tested that in cancer cell lines. We assume that the major discrepancy of expression rates of *MAGE* genes between other reports and our findings might result from this. Since RNA or DNA was extracted from surgically removed frozen tissue biopsies, the tumor tissues may have been contaminated with normal stromal cells, therefore, masking the true levels of hypomethylation or expression

Table 2 Summary of expressions, promoter methylation status and clinical associations of the *MAGE-A1* and *MAGE-A3* genes in 32 colorectal cancer cell lines and 87 colorectal cancer tissues

	% of expression		% of hypomethylation	
	MAGE-A1	*MAGE-A3*	*MAGE-A1*	*MAGE-A3*
Cancer cell lines	59	66	81	81
Cancer tissues	NT[1]	NT	43	77
Normal tissues[2]	NT	NT	2	6

	MAGE-A1 hypomethylation			*MAGE-A3* hypomethylation		
	+	-	*P*	+	-	*P*
Location						
Proximal	14 (31.1%)	31(68.9%)		34 (75.6%)	11 (24.4%)	
Distal	17 (56.7%)	13(43.3%)	0.028	26 (86.7%)	4 (13.3%)	0.239
Sex						
Male	17 (30.9%)	38(69.1%)		38 (69.1%)	17 (30.9%)	
Female	20 (62.5%)	12(18.4%)	0.004	29 (90.6%)	3 (9.4%)	0.021

[1]Not tested; [2]Corresponding normal tissues of cancer tissues; +: Represent hypomethylation of *MAGE* genes; -: Represent no hypomethylation of *MAGE* genes.

of the *MAGE* genes in cancer tissues[6]. For further analysis of the methylation status of the *MAGE* genes in colorectal cancer tissues, laser capture microdissection techniques would allow more precise isolation of cancer cells and normal cells. It has already been reported that cancer cell lines have much higher levels of CpG island hypermethylation than corresponding malignant tissues, which may explain our lower incidence of hypomethylation in tissues *versus* cell lines. Moreover, cancer cells might be clonally selected with growth advantages over cancer cell lines. However, cancer cell lines often preserve hypermethylation or hypomethylation from the tumors they originate, thus they are indeed useful tools to study methylation status.

We analyzed promoter unmethylation of the *MAGE-A1* and *MAGE-A3* genes with a methylation-specific PCR after sodium-bisulfite modification and by direct sequencing analysis. Of the 32 cell lines analyzed, the promoter hypomethylation of *MAGE-A1* and *MAGE-A3* was observed in both at 26 cell lines each. Further, 23 cell lines (SNU-61, SNU-175, SNU-503, SNU-769A, SNU-769B, SNU-1033, SNU-1197A, SNU-C1, SNU-C2A, Caco2, COLO201, COLO205, COLO320, DLD-1, HCT-15, HCT-116, HT-29, Lovo, LS174T, NCI-H716, SW403, SW1116, and WiDR) were simultaneously unmethylated in both the *MAGE-A1* and *MAGE-A3* genes. With exception, there were two cell lines (SNU-61, COLO201) with negative gene expression for either *MAGE-A1* or *MAGE-A3*, but unmethylated *MAGE-A1* or *MAGE-A3* promoter was detected. On the contrary, there were four cell lines (SNU-283, SNU-407, SNU-1047 and SW480) in which *MAGE* genes were strongly expressed, although no unmethylated *MAGE* promoter could be detected, suggesting the activation or inactivation of *MAGE* expression by another mechanism.

In our study, the rates of hypomethylation of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer cell lines were 81% in both and those in colorectal cancer tissues were 43% and 77%, respectively. The DNA was extracted from the surgically removed frozen-tissues; however, the tumor tissues might have been contaminated with some normal stromal cells. Therefore, the levels of hypomethylation of *MAGE-A1* and *MAGE- A3* genes in cancer tissues might be affected by the DNA from normal cells. To obtain a better understanding of the promoter hypomethylation status of the *MAGE-A1* and *MAGE-A3* genes in colorectal cancer tissues, expression analysis of *MAGE-A1* and *MAGE-A3* (such as, *in situ* hybridization or immunostaining) and the more precise methylation analysis method (such as, laser capture microdissection techniques) to isolate cancer cells from normal cells need to be performed. The hypomethylation of the *MAGE-A1* and *MAGE-A3* genes in corresponding normal tissues was detected in only 2 and 5 samples (2% and 6%), respectively (Table 2).

To evaluate the association between the clinical parameters and *MAGE* expression, the Pearson χ^2 test was used to evaluate differences in tumor location (proximal or distal) or gender, and significance was determined using 95% confidence intervals. In our study, unmethylated *MAGE-A1* DNA expression was significantly different in respect of tumor location and gender. Unmethylated *MAGE-A1* DNA expression was significantly higher in distal location ($P = 0.028$) and in females ($P = 0.004$). However, unmethylated *MAGE-A3* DNA expression was not significantly associated with tumor location ($P = 0.239$), while it was only related to female gender ($P = 0.021$).

Our results supported the role of the promoter methylation in maintaining a silent phenotype of the *MAGE-A1* and *MAGE-A3* genes, as the *MAGE* gene was re-expressed after treatment with 5-aza-2'-deoxycytidine. This agent reactivates gene expression when methylation of CpG islands is the cause of reduced gene expression. We demonstrated that the *MAGE-A1* and *MAGE-A3* mRNAs were re-expressed after 5-aza-2'-deoxycytidine treatment in all 8 and 9 cell lines that did not express *MAGE-A1* and *MAGE-A3* mRNAs, respectively. However, the SNU-1040 and COLO 205 cell lines did not show re-expression, suggesting the inactivation of *MAGE-A1* expression by another mechanism.

In conclusion, we observed hypomethylation in the promoter region of both the *MAGE-A1* and *MAGE-A3* genes in 23 of 32 colorectal cancer cell lines. This methylation was confirmed by MS-PCR, treatment with 5-aza-2'-deoxycytidine, and bisulfite direct sequencing analysis. Hypomethylation of the promoter region appears to be a frequent phenomenon in human colorectal cancers and upregulates transcription of the *MAGE-A1 and MAGE-A3* genes in colorectal cancer cells. In addition, out of 87 colorectal cancer tissues, we observed hypomethylation in the promoter regions of the *MAGE-A1* and *MAGE-A3* genes in 37 (43%) and 67 (77%) tissues, respectively. This suggests that promoter hypomethylation of *MAGE-A1* and *MAGE-A3* genes up-regulates its expression in colorectal carcinomas as well as in gastric cancers, and might play a significant role in the development and progression of human colorectal carcinomas.

REFERENCES

1 **Zöchbauer-Müller S**, Fong KM, Maitra A, Lam S, Geradts J,

Ashfaq R, Virmani AK, Milchgrub S, Gazdar AF, Minna JD. 5′ CpG island methylation of the FHIT gene is correlated with loss of gene expression in lung and breast cancer. *Cancer Res* 2001; **61**: 3581-3585

2 **Song SH**, Jong HS, Choi HH, Inoue H, Tanabe T, Kim NK, Bang YJ. Transcriptional silencing of Cyclooxygenase-2 by hyper-methylation of the 5′ CpG island in human gastric carcinoma cells. *Cancer Res* 2001; **61**: 4628-4635

3 **Suh ER**, Ha CS, Rankin EB, Toyota M, Traber PG. DNA methylation down-regulates CDX1 gene expression in colorectal cancer cell lines. *J Biol Chem* 2002; **277**: 35795-35800

4 **Melki JR**, Vincent PC, Brown RD, Clark SJ. Hypermethylation of E-cadherin in leukemia. *Blood* 2000; **95**: 3208-3213

5 **Yang Q**, Nakamura M, Nakamura Y, Yoshimura G, Suzuma T, Umemura T, Shimizu Y, Mori I, Sakurai T, Kakudo K. Two-hit inactivation of FHIT by loss of heterozygosity and hypermethylation in breast cancer. *Clin Cancer Res* 2002; **8**: 2890-2893

6 **Ku JL**, Kang SB, Shin YK, Kang HC, Hong SH, Kim IJ, Shin JH, Han IO, Park JG. Promoter hypermethylation downregulates RUNX3 gene expression in colorectal cancer cell lines. *Oncogene* 2004; **23**: 6736-6742

7 **Feinberg AP**, Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. *Biochem Biophys Res Commun* 1983; **111**: 47-54

8 **Fang JY**, Zhu SS, Xiao SD, Jiang SJ, Shi Y, Chen XY, Zhou XM, Qian LF. Studies on the hypomethylation of c-myc, c-Ha-ras oncogenes and histopathological changes in human gastric carcinoma. *J Gastroenterol Hepatol* 1996; **11**: 1079-1082

9 **Van Der Bruggen P**, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, Chapiro J, Van Den Eynde BJ, Brasseur F, Boon T. Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol Rev* 2002; **188**: 51-64

10 **De Smet C**, Lurquin C, Lethé B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol* 1999; **19**: 7327-7335

11 **De Smet C**, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. *Proc Natl Acad Sci USA* 1996; **93**: 7149-7153

12 **Honda T**, Tamura G, Waki T, Kawata S, Terashima M, Nishizuka S, Motoyama T. Demethylation of MAGE promoters during gastric cancer progression. *Br J Cancer* 2004; **90**: 838-843

13 **Liu J**, Wang G, Okutomi T, Chen Z. Expression of MAGE-A1 and MAGE-A3 genes in human salivary gland carcinomas. *Chin Med J* (Engl) 2003; **116**: 897-900

14 **Park JG**, Oie HK, Sugarbaker PH, Henslee JG, Chen TR, Johnson BE, Gazdar A. Characteristics of cell lines established from human colorectal carcinoma. *Cancer Res* 1987; **47**: 6710-6718

15 **Oh JH**, Ku JL, Yoon KA, Kwon HJ, Kim WH, Park HS, Yeo KS, Song SY, Chung JK, Park JG. Establishment and characterization of 12 human colorectal-carcinoma cell lines. *Int J Cancer* 1999; **81**: 902-910

16 **Herman JG**, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821-9826

17 **De Smet C**, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′region of gene MAGE-A1 in tumor cells. *Mol Cell Biol* 2004; **24**: 4781-4790

18 **Park MS**, Park JW, Jeon CH, Lee KD, Chang HK. Expression of melanoma antigen-encoding genes (MAGE) by common primers for MAGE-A1 to -A6 in colorectal carcinomas among Koreans. *J Korean Med Sci* 2002; **17**: 497-501

19 **Koketsu S**, Watanabe T, Kazama S, Ishihara S, Komuro Y, Nagawa H. What types of colorectal cancer overexpress the MAGE protein? *Hepatogastroenterology* 2004; **51**: 1648-1652

20 **Xiao J**, Chen HS, Fei R, Cong X, Wang LP, Wang Y, Jiang D, Wei L, Wang Y. Expression of MAGE-A1 mRNA is associated with gene hypomethylation in hepatocarcinoma cell lines. *J Gastroenterol* 2005; **40**: 716-721

21 **Zendman AJ**, de Wit NJ, van Kraats AA, Weidle UH, Ruiter DJ, van Muijen GN. Expression profile of genes coding for melanoma differentiation antigens and cancer/testis antigens in metastatic lesions of human cutaneous melanoma. *Melanoma Res* 2001; **11**: 451-459

22 **Li M**, Yuan YH, Han Y, Liu YX, Yan L, Wang Y, Gu J. Expression profile of cancer-testis genes in 121 human colorectal cancer tissue and adjacent normal tissue. *Clin Cancer Res* 2005; **11**: 1809-1814

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H pylori infection and reflux oesophagitis: A case-control study

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Abstract

AIM: To examine the relationship between *H pylori* and gastro-oesophageal reflux disease (GORD) in Iran.

METHODS: In this study 51 GORD patients (referred to endoscopy at Taleghani hospital) were compared with 49 age-sex matched controls. Diagnosis of *H pylori* was made by gastric mucosal biopsy and rapid urease test (positive if the result of one or both diagnostic methods was positive). Updated Sydney system was used to report histopathological changes.

RESULTS: The frequency of *H pylori* infection based on rapid urease test and histology was 88.2% (45) in patients and 77.6% (38) in controls, which showed no significant difference. The frequency of *H pylori* infection was significantly higher in the antrum than in the corpus and cardia. The mean activity, inflammation, and gastritis scores were also higher in the antrum of patients than in the antrum of controls. The mean scores were significantly higher in the corpus of controls than in the corpus of patients. Diffuse active gastritis was observed in a significantly larger number of controls, while the frequency of diffuse chronic gastritis was higher in patients. There was no significant difference in the frequency of other histological findings between patients and controls.

CONCLUSION: *H pylori* infection cannot prevent GORD in this region.

Key words: *H pylori*; Gastro-oesophageal reflux diseases; Reflux oesophagitis

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INTRODUCTION

Heartburn is a common symptom in the general population[1,2] and is associated with the development of adenocarcinoma of the oesophagus and cardia[3]. Gastritis-associated hypochlorhydria may protect against gastro-oesophageal reflux disease (GORD)[4,5]. It has hypothesized that the declined *H pylori* infection results in a decline in peptic ulcer and a concomitant increase in reflux disease and associated oesophageal adenocarcinoma[6,7]. However, the relationship between *H pylori* infection and GORD has not been established[8-11]. It was reported that prospective, large studies are needed to explore the *H pylori*-gastro-oesophageal disease relationship further and to avoid confusing potential benefits with known risks[9-12].

The main of this study was to investigate whether there is a difference between the frequencies of *H pylori* infection in cases and controls, and the possible relationship between *H pylori* infection and GORD.

MATERIALS AND METHODS

Patients

In this study, patients with a history of heartburn, at least two times a week for a period of more than 3 mo, referred for gastrointestinal endoscopy at Taleghani Hospital, Shaheed Beheshti University of Medical Sciences, between March 2001 and February 2002, were enrolled. The reflux oesophagitis group included 51 patients (31 men and 20 women, mean age, 54.1 ± 17.2 years, range 17-80 years) with endoscopically diagnosed erosive reflux oesophagitis.

Table 1 Frequency of *H pylori* infection in two groups *n* (%)

Group	*H pylori*			Rut	Diagnosis of *H pylori*	Total
	Cardia	Corpus	Antrum			
Case	27 (52.9)	25 (49.0)	37 (72.5)	23 (45.1)	45 (88.2)	51 (100.0)
L.A. classification						
A	11 (21.6)	8 (15.7)	12 (23.5)	9 (17.7)	16 (31.4)	17 (33.3)
B	11 (21.6)	11 (21.6)	17 (33.4)	12 (23.5)	21 (41.2)	24 (47.1)
C	4 (7.8)	5 (9.8)	7 (13.7)	2 (3.9)	7 (13.7)	9 (17.7)
D	1 (1.9)	1 (1.9)	1 (1.9)	0	1 (1.9)	1 (1.9)
Control	28 (57.1)	27 (55.1)	31 (63.3)	22 (44.9)	38 (77.6)	49 (100.0)
Total	55 (55.0)	52 (52.0)	68 (68.0)	45 (45.0)	83 (83.0)	100 (100.0)

Patients with a history of upper gastrointestinal (GI) surgery, malignancy, oesophageal varices, and antibiotics or bismuth consumption during the last 6 mo, together with those using H_2 blockers, proton pump inhibitors (PPIs), alcohol, or non steroidal anti inflammatory drugs (NSAIDs) during the last 4 wk, were excluded from the study. The control group comprised: 49 asymptomatic patients (29 men and 20 women, mean age, 52.2 ± 17.1 years, range 18-80 years) without reflux oesophagitis, any symptom of upper GI diseases, and any lesion in their endoscopy, which was performed for other reasons (work up for iron deficiency, possible malignancy, and ERCP, sphincterotomy, or stone extraction candidates). Two cases of control group were missed during the study. The cases and controls were sex and age matched with a maximum difference of less than 3 years. Written informed consent was obtained for all upper endoscopy and biopsy procedures. This study was approved by the Ethics Committee of the Research Center of Gastroenterology and Liver Diseases, Shaheed Beheshti University of Medical Sciences.

Endoscopy and gastric biopsies

Endoscopy was performed for both case and control groups, by two endoscopists blinded to the status of the controls and patients. The presence and grading of reflux oesophagitis were determined according to L.A. classification, from A (least severe) to D (most severe)[13]. During endoscopy, two biopsies were taken from the antrum, corpus, and cardia, and stained with standard haematoxylin/eosin and Geimsa to identify *H pylori* and histopathological changes. Rapid urease test was performed on the biopsy specimens from antrum, corpus, and cardia. The urease test was considered positive when the urea solution changed from yellow to pink at room temperature within 24 h. The diagnosis of *H pylori* infection was made by positive findings on either histology or urease test. Patients were considered to be *H pylori*-positive if the result of one or both diagnostic methods was positive and *H pylori*-negative if both methods revealed negative results.

For histopathological analysis, biopsy specimens were fixed in 40 g/L neutral-buffered formaldehyde and embedded in paraffin. Five-micron thick sections were cut from each paraffin block and stained with haematoxylin and eosin for routine histology. Two pathologists blinded to the clinical information of subjects assessed the histopathological changes independently. Updated Sydney system was used to report histopathological changes[14]. The degrees of inflammation and activity were scored from 0 (absent) to 3 (most severe). The inflammation score and activity score were summed and expressed as the gastritis score. The predominance for the anatomic regions in gastritis was determined based on the degree of inflammation in the different anatomic parts of the stomach. If the degree of inflammation was higher, the anatomic place with a higher grade of inflammation was stated as the predominant region. For the diagnosis of multifocal atrophic gastritis, we determined intestinal metaplasia or significant mucosal atrophy. Because of the low reproducibility for routine grading of mucosal atrophy, atrophy score was not used as a marker for grading[14].

Statistical analysis

Results were expressed as mean ± SD. Odd's ratios (95% CI) were calculated to evaluate the differences in the frequency of *H pylori* infection and other histological findings between patient and control groups. Cochran's Q test was used to compare the frequency of *H pylori* infection in the cardia, corpus, and antrum. Mann-Whitney *U* test was used to analyze the differences in activity, inflammation, and gastritis scores between the two groups and Friedman test was used to analyze the different scores in the cardia, corpus, and antrum. $P < 0.05$ was considered statistically significant.

A sample size of about 50 for patient and control groups was considered to have an 80% detecting rate (at the two-sided 5% level) with at least a 25% difference in the prevalence of *H pylori* between the two groups.

RESULTS

Among the 51 reflux oesophagitis patients, 17 (33.3%) were in grade A, 24 (47.1%) in grade B, 9 (17.6%) in grade C, and 1 (1.9%) in grade D (Table 1). Hiatal hernia was observed in 30 (58.8%) patients. The prevalence of *H pylori* infection is shown in Table 1. The frequency of *H pylori* in the antrum was significantly higher than that in the corpus and cardia of the patients ($P < 0.01$), while the differences were not significant in different regions of stomach of the controls, which might be due to the inadequate sample size. We were not able to find any significant difference in the frequency of *H pylori* infection between the two groups (OR: 2.2, 95% CI: 0.7-7.4) as shown in Table 2.

The different histological findings in patients and

Table 2 Different histological findings in two groups *n* (%)

Histological finding	Case	Control	Total	OR (95% CI)
H pylori infection[1]	45 (88.2)	38 (77.6)	83 (83.0)	2.2 (0.7-7.4)
Chronic inflammation in cardia	14 (27.5)	18 (36.7)	32 (32.0)	0.7 (0.3-1.7)
Chronic inflammation in corpus	8 (15.7)	22 (44.9)	30 (30.0)	0.2 (0.1-0.6)
Chronic inflammation in antrum	28 (54.9)	30 (61.2)	58 (58.0)	0.8 (0.3-1.8)
Overall gastritis categorization (Sydney classification)				
Diffuse chronic active gastritis	15 (29.4)	26 (53.1)	41 (42.0)	0.4 (0.2-0.9)
Cardia predominant chronic active gastritis	1 (2.0)	1 (2.0)	2 (2.0)	0.9 (0.0-36.3)
Corpus predominant chronic active gastritis	0	1 (2.0)	1 (1.0)	0.0 (0.0-16.9)
Antrum predominant chronic active gastritis	3 (5.9)	5 (10.2)	8 (8.0)	0.6 (0.1-2.9)
Multifocal metaplastic or atrophic gastritis	1 (2.0)	1 (2.0)	2 (2.0)	0.9 (0.0-36.3)
Chronic carditis	1 (2.0)	2 (4.1)	3 (3.0)	0.5 (0.0-6.9)
Diffuse chronic gastritis	10 (19.6)	2 (4.1)	12 (12.0)	5.7 (1.1-40.4)
Normal	20 (39.2)	11 (22.4)	31 (31.0)	2.2 (0.9-5.8)
Total	51 (100.0)	49 (100.0)	100 (100.0)	-

[1]Based on histology and rapid urease test.

controls are shown in Table 2. The frequency of chronic inflammation in the corpus was significantly higher in controls than in patients (OR: 0.2, 95% CI: 0.1-0.6). Diffuse active gastritis was also observed in controls (OR: 0.4, 95% CI: 0.2-0.9), while diffuse chronic gastritis was observed in patients (OR: 5.7, 95% CI: 1.1-40.4). The frequency of intestinal metaplasia and mucosal atrophy was not significantly different between the two groups (Table 2).

The inflammation, activity, and gastritis scores in both groups are depicted in Table 3 and Figure 1. The mean activity score in the cardia, corpus, and antrum of controls was significantly higher than that of patients ($P < 0.01$ or $P < 0.001$, Figure 1A). The inflammation score was higher in the corpus of controls than that in patients ($P < 0.01$, Figure 1B), while the inflammation score of the cardia and antrum was not significantly different between the two groups. Similarly gastritis score was significantly higher in controls than in patients ($P < 0.01$ and $P < 0.05$, Figure 1C).

The mean activity score was significantly higher in the antrum than in the corpus and cardia of controls ($P < 0.001$, Table 3), while the differences were not significant in patients probably due to the inadequate sample size. The mean inflammation and gastritis scores were also significantly higher in antrum than in corpus and cardia of both patients and controls ($P < 0.001$, Table 3). These findings together with the higher frequency of *H pylori* infection in the antrum indicated that *H pylori* in antrum could induce inflammation.

Table 3 Scores of inflammation, activity, and gastritis in two groups

Group	Cardia	Corpus	Antrum	*P*
	Median (range)			
Case				
H pylori n (%)	27 (52.9)	25 (49.0)	37 (72.5)	0.005[1]
Activity score	0 (0-2)	0 (0-2)	0 (0-3)	0.057[2]
Inflammation score	1 (0-2)	1 (0-2)	2 (0-3)	0.000[2]
Gastritis score	0 (0-4)	1 (0-4)	2 (0-6)	0.000[2]
Control				
H pylori n (%)	28 (57.1)	27 (55.1)	31 (63.3)	0.465[1]
Activity score	1 (0-2)	1 (0-3)	1 (0-3)	0.000[2]
Inflammation score	1 (0-3)	1 (0-3)	2 (0-3)	0.000[2]
Gastritis score	2 (0-5)	2 (0-6)	3 (0-6)	0.000[2]

[1]Cochran's Q test; [2]Friedman test.

DISCUSSION

Increasing attention has been paid to the relationship between *H pylori* infection and reflux oesophagitis in recent years. GORD is a common condition affecting 25%-40% of the population[2]. The presence of hiatal hernia[15], transient relaxation of the lower oesophageal sphincter[16,17], and impaired clearance of regurgitated gastric contents in the oesophagus[18] are considered possible causative factors for GORD.

There is evidence that infection with *H pylori* is the principal cause of peptic ulcer disease[10]. However, there is uncertainty about the role of this organism in GORD and the available data do not demonstrate an evident association between these two factors, although an etiologic link has been found between *H pylori* infection and GORD or peptic oesophagitis[10]. The prevalence of *H pylori* infection in patients with GORD in our study (88.2%) was higher than that reported in other studies[9], suggesting that *H pylori* infection is more frequent in developing countries than in industrialized countries[19].

No difference was found in the prevalence of *H pylori* between patients with reflux oesophagitis and controls in this study. Conflicting evidence about the association of *H pylori* infection with GORD has been reported and geographical location is an important determinant[9]. The pathogenic role of *H pylori* in reflux oesophagitis is suspected in earlier studies[20] while other studies have found no relationship between *H pylori* prevalence in GORD patients with that reported in other patients[10,21-34]. In contrast, the possible protective role of *H pylori* in reflux oesophagitis and other GORD-related diseases such as Barrett's oesophagus and oesophageal adenocarcinoma has recently been suggested[35-39]. *H pylori* can cause chronic gastritis in virtually all infected people. This persistent inflammation ultimately leads to loss of the normal architecture of gastric mucosa, disappearance of gastric

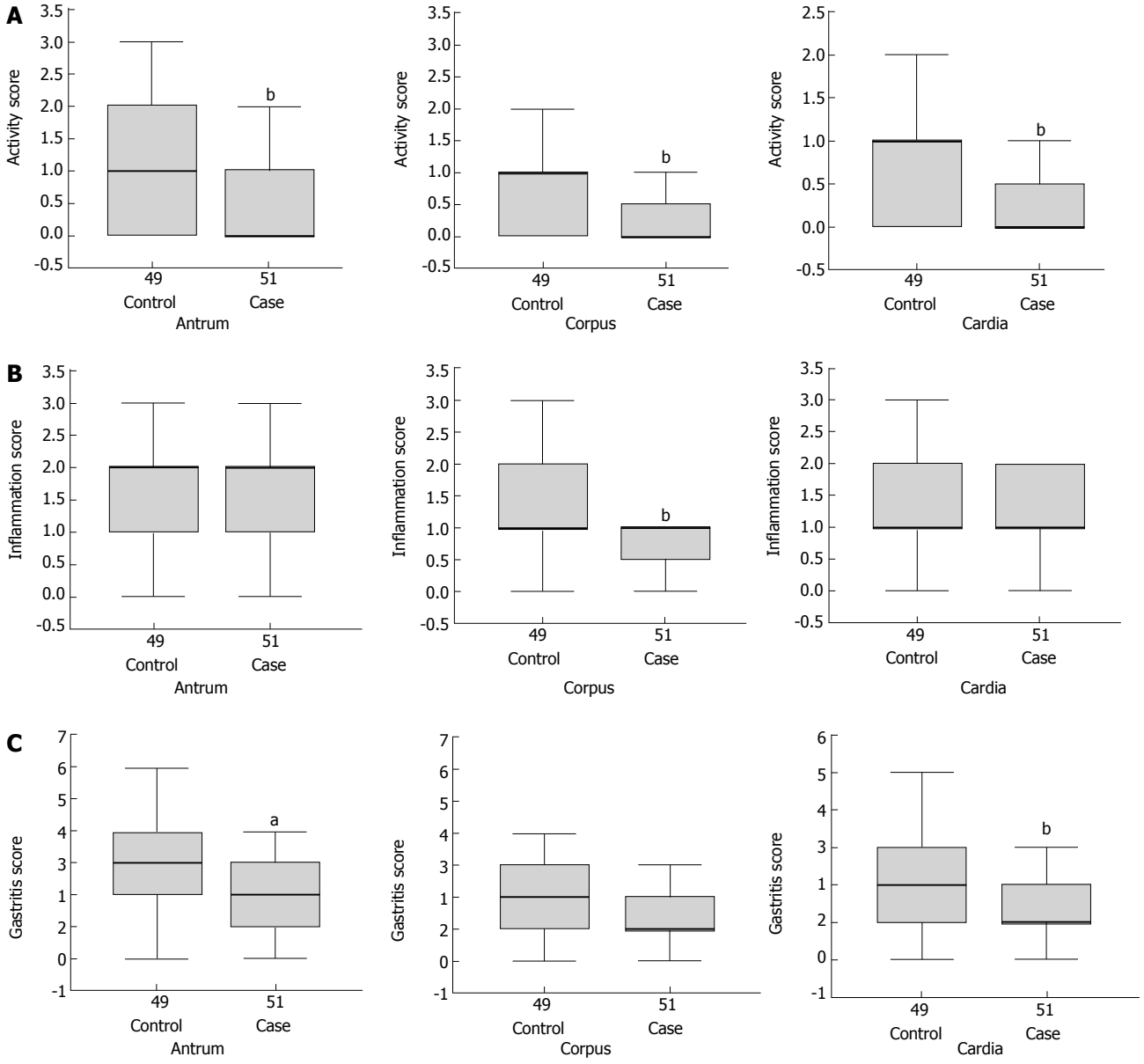

Figure 1 Scores of activity (**A**), inflammation (**B**), and gastritis (**C**) in the antrum and corpus of patients and controls. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* controls.

glands and specialized cells[40]. Diffuse active gastritis was observed in controls while diffuse chronic gastritis was observed in patients in the present study, suggesting that active inflammation might play a protective role in GORD. Chronic antrum-predominant gastritis has been shown to be associated with secretion of acid and formation of duodenal ulcer[8,41]. In patients otherwise predisposed to reflux disease, antrum-predominant gastritis may therefore increase acid production and reflux disease development[8]. On the other hand, atrophy induced by chronic *H pylori* infection (chronic corpus gastritis) with decreased gastric acid production can protect against reflux oesophagitis[8,11,42]. As a consequence, the antrum predominant inflammation might be considered a factor for *H pylori* infection.

In conclusion, *H pylori* infection is not associated with DORD. Multicentre prospective studies with a larger sample size are needed to explore the relationship between *H pylori* infection and DORD.

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REFERENCES

1 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456
2 **Jones R**. Gastro-oesophageal reflux disease in general practice. *Scand J Gastroenterol Suppl* 1995; **211**: 35-38
3 **Lagergren J**, Bergström R, Lindgren A, Nyrén O. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N Engl J Med* 1999; **340**: 825-831
4 **Graham DY**, Yamaoka Y. H. pylori and cagA: relationships with gastric cancer, duodenal ulcer, and reflux esophagitis and its complications. *Helicobacter* 1998; **3**: 145-151
5 **Richter JE**, Falk GW, Vaezi MF. Helicobacter pylori and

gastroesophageal reflux disease: the bug may not be all bad. *Am J Gastroenterol* 1998; **93**: 1800-1802
6 **el-Serag HB**, Sonnenberg A. Opposing time trends of peptic ulcer and reflux disease. *Gut* 1998; **43**: 327-333
7 **Pera M**, Cameron AJ, Trastek VF, Carpenter HA, Zinsmeister AR. Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. *Gastroenterology* 1993; **104**: 510-513
8 **Gisbert JP**, Pajares JM, Losa C. Helicobacter pylori and gastroesophageal reflux disease: friends or foes? *Hepatogastroenterology* 1999; **46**: 1023-1029
9 **Raghunath A**, Hungin AP, Wooff D, Childs S. Prevalence of Helicobacter pylori in patients with gastro-oesophageal reflux disease: systematic review. *BMJ* 2003; **326**: 737
10 **Gisbert JP**, de Pedro A, Losa C, Barreiro A, Pajares JM. Helicobacter pylori and gastroesophageal reflux disease: lack of influence of infection on twenty-four-hour esophageal pH monitoring and endoscopic findings. *J Clin Gastroenterol* 2001; **32**: 210-214
11 **Vakil NB**. Review article: gastro-oesophageal reflux disease and Helicobacter pylori infection. *Aliment Pharmacol Ther* 2002; **16** Suppl 1: 47-51
12 **Malfertheiner P**, O'Connor HJ, Genta RM, Unge P, Axon AT. Symposium: Helicobacter pylori and clinical risks--focus on gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 2002; **16** Suppl 3: 1-10
13 **Armstrong D**, Bennett JR, Blum AL, Dent J, De Dombal FT, Galmiche JP, Lundell L, Margulies M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. The endoscopic assessment of esophagitis: a progress report on observer agreement. *Gastroenterology* 1996; **111**: 85-92
14 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181
15 **ALLISON PR**. Reflux esophagitis, sliding hiatal hernia, and the anatomy of repair. *Surg Gynecol Obstet* 1951; **92**: 419-431
16 **Dodds WJ**, Dent J, Hogan WJ, Helm JF, Hauser R, Patel GK, Egide MS. Mechanisms of gastroesophageal reflux in patients with reflux esophagitis. *N Engl J Med* 1982; **307**: 1547-1552
17 **Dent J**, Holloway RH, Toouli J, Dodds WJ. Mechanisms of lower oesophageal sphincter incompetence in patients with symptomatic gastrooesophageal reflux. *Gut* 1988; **29**: 1020-1028
18 **Stanciu C**, Bennett JR. Oesophageal acid clearing: one factor in the production of reflux oesophagitis. *Gut* 1974; **15**: 852-857
19 **Feldman RA**, Eccersley AJ, Hardie JM. Epidemiology of Helicobacter pylori: acquisition, transmission, population prevalence and disease-to-infection ratio. *Br Med Bull* 1998; **54**: 39-53
20 **Borkent MV**, Beker JA. Treatment of ulcerative reflux oesophagitis with colloidal bismuth subcitrate in combination with cimetidine. *Gut* 1988; **29**: 385-389
21 **Rosioru C**, Glassman MS, Halata MS, Schwarz SM. Esophagitis and Helicobacter pylori in children: incidence and therapeutic implications. *Am J Gastroenterol* 1993; **88**: 510-513
22 **Werdmuller BF**, Loffeld RJ. Helicobacter pylori infection has no role in the pathogenesis of reflux esophagitis. *Dig Dis Sci* 1997; **42**: 103-105
23 **Walker SJ**, Birch PJ, Stewart M, Stoddard CJ, Hart CA, Day DW. Patterns of colonisation of Campylobacter pylori in the oesophagus, stomach and duodenum. *Gut* 1989; **30**: 1334-1338
24 **Cheng EH**, Bermanski P, Silversmith M, Valenstein P, Kawanishi H. Prevalence of Campylobacter pylori in esophagitis, gastritis, and duodenal disease. *Arch Intern Med* 1989; **149**: 1373-1375
25 **Befrits R**, Granström M, Rylander M, Rubio C. Helicobacter pylori in 205 consecutive endoscopy patients. *Scand J Infect Dis* 1993; **25**: 185-191
26 **O'Connor HJ**, Cunnane K. Helicobacter pylori and gastro-oesophageal reflux disease--a prospective study. *Ir J Med Sci* 1994; **163**: 369-373
27 **Boixeda D**, Gisbert JP, Cantón R, Alvarez Baleriola I, Gil Grande LA, Martín de Argila C. [Is there any association between Helicobacter pylori infection and peptic esophagitis?]. *Med Clin* (Barc) 1995; **105**: 774-777
28 **Liston R**, Pitt MA, Banerjee AK. Reflux oesophagitis and Helicobacter pylori infection in elderly patients. *Postgrad Med J* 1996; **72**: 221-223
29 **Newton M**, Bryan R, Burnham WR, Kamm MA. Evaluation of Helicobacter pylori in reflux oesophagitis and Barrett's oesophagus. *Gut* 1997; **40**: 9-13
30 **Csendes A**, Smok G, Cerda G, Burdiles P, Mazza D, Csendes P. Prevalence of Helicobacter pylori infection in 190 control subjects and in 236 patients with gastroesophageal reflux, erosive esophagitis or Barrett's esophagus. *Dis Esophagus* 1997; **10**: 38-42
31 **Hackelsberger A**, Schultze V, Günther T, von Arnim U, Manes G, Malfertheiner P. The prevalence of Helicobacter pylori gastritis in patients with reflux oesophagitis: a case-control study. *Eur J Gastroenterol Hepatol* 1998; **10**: 465-468
32 **Ho KY**, Kang JY. Reflux esophagitis patients in Singapore have motor and acid exposure abnormalities similar to patients in the Western hemisphere. *Am J Gastroenterol* 1999; **94**: 1186-1191
33 **Pieramico O**, Zanetti MV. Relationship between intestinal metaplasia of the gastro-oesophageal junction, Helicobacter pylori infection and gastro-oesophageal reflux disease: a prospective study. *Dig Liver Dis* 2000; **32**: 567-572
34 **Oberg S**, Peters JH, Nigro JJ, Theisen J, Hagen JA, DeMeester SR, Bremner CG, DeMeester TR. Helicobacter pylori is not associated with the manifestations of gastroesophageal reflux disease. *Arch Surg* 1999; **134**: 722-726
35 **Labenz J**, Blum AL, Bayerdörffer E, Meining A, Stolte M, Börsch G. Curing Helicobacter pylori infection in patients with duodenal ulcer may provoke reflux esophagitis. *Gastroenterology* 1997; **112**: 1442-1447
36 **Vicari JJ**, Peek RM, Falk GW, Goldblum JR, Easley KA, Schnell J, Perez-Perez GI, Halter SA, Rice TW, Blaser MJ, Richter JE. The seroprevalence of cagA-positive Helicobacter pylori strains in the spectrum of gastroesophageal reflux disease. *Gastroenterology* 1998; **115**: 50-57
37 **Xia HH**, Talley NJ. Helicobacter pylori infection, reflux esophagitis, and atrophic gastritis: an unexplored triangle. *Am J Gastroenterol* 1998; **93**: 394-400
38 **Chow WH**, Blaser MJ, Blot WJ, Gammon MD, Vaughan TL, Risch HA, Perez-Perez GI, Schoenberg JB, Stanford JL, Rotterdam H, West AB, Fraumeni JF Jr. An inverse relation between cagA+ strains of Helicobacter pylori infection and risk of esophageal and gastric cardia adenocarcinoma. *Cancer Res* 1998; **58**: 588-590
39 **Yamaji Y**, Mitsushima T, Ikuma H, Okamoto M, Yoshida H, Kawabe T, Shiratori Y, Saito K, Yokouchi K, Omata M. Inverse background of Helicobacter pylori antibody and pepsinogen in reflux oesophagitis compared with gastric cancer: analysis of 5732 Japanese subjects. *Gut* 2001; **49**: 335-340
40 **Kuipers EJ**, Lundell L, Klinkenberg-Knol EC, Havu N, Festen HP, Liedman B, Lamers CB, Jansen JB, Dalenback J, Snel P, Nelis GF, Meuwissen SG. Atrophic gastritis and Helicobacter pylori infection in patients with reflux esophagitis treated with omeprazole or fundoplication. *N Engl J Med* 1996; **334**: 1018-1022
41 **Labenz J**, Malfertheiner P. Helicobacter pylori in gastro-oesophageal reflux disease: causal agent, independent or protective factor? *Gut* 1997; **41**: 277-280
42 **Koike T**, Ohara S, Sekine H, Iijima K, Abe Y, Kato K, Toyota T, Shimosegawa T. Helicobacter pylori infection prevents erosive reflux oesophagitis by decreasing gastric acid secretion. *Gut* 2001; **49**: 330-334

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Natural maternal transmission of *H pylori* in Mongolian gerbils

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Abstract

AIM: To investigate maternal *H pylori* infection status to determine the potential of maternal transmission.

METHODS: In the present study, we examined these issues in an experimental murine model, which is a Mongolian gerbil model that has been reported as an optimal laboratory animal model to study *H pylori*. Pregnant Mongolian gerbils, infected experimentally with *H pylori*, were divided into as four groups. Following the experimental design, the stomachs of the mother and litters were isolated and assessed for transmission of *H pylori* at the prenatal period, parturition day, 1-wk old and 3-wk old respectively. Bacterial culture and polymerase chain reaction (PCR) were used to examine the presence of transmitted *H pylori*.

RESULTS: All litters showed no transmission of *H pylori* during pregnancy and at parturition day. However, they revealed 33.3% and 69.6% at 1-wk and 3-wk of age respectively by PCR.

CONCLUSION: These results suggested that vertical infection during the prenatal period or delivery procedure is unlikely as a route of mother-to-child *H pylori* infection. It may be that *H pylori* is acquired through breast-feeding, contaminated saliva and fecal-oral transmission during co-habitation.

Key words: *H pylori*; Vertical; Maternal; Transmission

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INTRODUCTION

H pylori is a gram-negative, spiral-shaped, microaerophilic bacterium that infects the human gastric mucosa[1]. Chronic infection is thought to be associated with chronic active gastritis, peptic ulcer and gastric malignancies, such as mucosa-associated B cell lymphoma and adenocarcinoma[2-4]. In particular, this organism has been categorized as a class I carcinogen by the World Health Organization[5] and previous studies have confirmed that long-term infection with *H pylori* induces adenocarcinoma in Mongolian gerbils[6,7]. In-depth knowledge of the transmission patterns may constitute important information for future intervention strategies. In the absence of consistent and verified environmental reservoirs, a predominantly person-to-person transmission has been postulated. *H pylori* infection is associated with poor living conditions, and possible transmission routes are fecal-oral, oral-oral, or gastro-oral, but firm evidence is lacking[8-11]. Young children are particularly vulnerable to infection by transmission of *H pylori* from their infected parents, especially infected mothers[12-15], and it is generally believed that such transmission is influenced by socio-economic status[16,17]. However, little is known about how and when maternal transmission occurs during the perinatal period, especially whether this occurs before or after parturition. In the present study, we examined these issues in an experimental murine model, which is a Mongolian gerbil model that has been reported as an optimal laboratory animal model to study *H pylori in vivo*[18].

The present study was designed to examine the incidence of vertical transmission of *H pylori* from their infected mother during the perinatal period in an experimental murine model.

MATERIALS AND METHODS

Experimental design

The experimental scheme of this study was summarized in Figure 1. Pregnant Mongolian gerbils were infected experimentally with *H pylori*. The stomachs of the litters were isolated and assessed for transmission of *H pylori* during pregnancy and at parturition day, 1 wk and 3 wk after delivery respectively. Their mother was also evaluated for the infectious status of *H pylori*. To determine the vertical transmission of *H pylori*, bacterial culture assay and polymerase chain reaction (PCR) were conducted with each sample.

Animals

Specific pathogen-free (SPF) 3-mo-old male and female

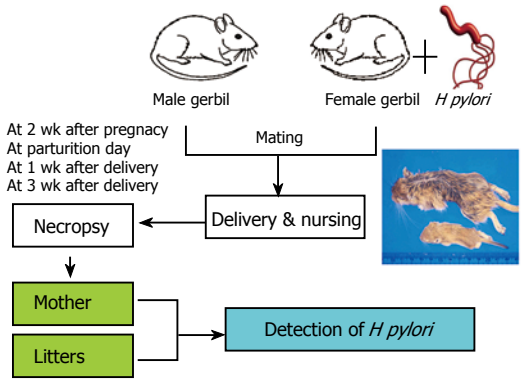

Figure 1 Scheme of experimental vertical transmission.

Figure 2 The mother and her litters were housed in one cage per family. For 12 h before their sacrifice, they were housed in grated cage and deprived diet.

Mongolian gerbils (*Meriones unguiculatus*) were obtained from the SPF Animal Facilities of College of Medicine, Seoul National University, South Korea. All animals were kept in the inspecting facility of Wonkwang University (Iksan, South Korea) for 1 wk before experimentation to allow acclimation. Thereafter, they were kept in an isolated SPF barrier room with regulated temperature (23℃ ± 1℃), humidity (50% ± 5%) and light/dark cycle (12/12 h). The animals were fed a sterilized pellet diet by 2 M rad radiation (Purina, Korea) and sterilized water *ad libitum*. All studies were performed in accordance with the Guide for Animal Experimentation by Wonkwang University and approved by the Institutional Animal Care and Use Committee of Wonkwang University (Iksan, South Korea). All efforts were made to minimize pain or discomfort of animals used.

Preparation of H pylori & inoculation

H pylori (ATCC 43504; American Type Culture Collection, USA) was incubated in a brain-heart infusion broth containing 10% fetal bovine serum at 37℃ overnight under a microaerophilic atmosphere and allowed to grow to a density of 2.0×10^9 colony-forming units (CFU) per 1 mL of culture broth. Animals were inoculated twice at 3-d intervals by oral administration of 1.0×10^9 CFU of *H pylori* suspended in 0.5 mL of broth. The challenged animals were confirmed to be *H pylori*-positive by PCR of their fecal samples as described previously[19]. *H pylori*-negative animals were excluded from the following study.

Maternal transmission of H pylori

One week after the challenge with *H pylori*, the infected females and males were transferred to separate cages for mating. As soon as a female was confirmed to be pregnant, she was separated from the group and cared for until delivery. Twelve infected pregnant females were used to determine maternal transmission. Three mothers were sacrificed at 2 wk after pregnancy. The gastric samples of mother and fetuses were isolated and submitted to determine *H pylori* infection. Also, another nine pregnant gerbils were cared for until delivery and the mother and her litters were housed in one cage per family. For 12 h before their sacrifice, they were housed in a grated cage and deprived diet (Figure 2). The stomachs of the mother and litters were isolated and assessed for transmission of *H pylori* at parturition day, 1 wk and 3 wk after delivery respectively. For the negative control, an uninfected female and her litters were sacrificed at 2 wk after pregnancy, parturition day, 1 wk and 3 wk after delivery respectively. Thereafter their gastric samples were submitted to examine *H pylori* infection.

Isolation of H pylori

Aliquots of homogenate were cultured on M-BHM pylori agar medium plates and the plates were incubated under the previous described condition[19]. To confirm *H pylori* infection, the remainder of the homogenate was used for the following PCR procedure.

Polymerase Chain Reaction

Bacterial DNAs were extracted from the above homogenate by bead beater-phenol extraction method[19]. Each sample homogenate was suspended in 200 μL of Tris-EDTA-NaCl buffer [10 mmol/L Tris-HCl, 1 mmol/L EDTA, and 100 mmol/L NaCl (pH 8.0)]. A bacterial suspension was placed in a 2.0-mL screw-cap microcentrifuge tube filled with 100 μL (packed volume) of glass beads (diameter, 0.1 mm; Biospec Products, USA) and 100 μL of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma Chemical Co., USA). The tube was oscillated on a Mini-Bead Beater (Biospec Products) for 1 min and was centrifuged (12000 × *g*, 5 min) to separate the phases. The aqueous phase was subsequently transferred into another clean tube; 10 μL of 3 mol/L sodium acetate and 250 μL of ice-cold absolute ethanol were added. To precipitate the DNA, the mixture was kept at -20℃ for 10 min. The harvested DNA pellets were dissolved in 60 μL of Tris-EDTA buffer [10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 8.0)] and were used as a template DNA for PCR. A set of primers (HF, 5'-ACTTTAAACGCATGAA GATAT-3'; and HR, 5'-ATATTTTGACCTTCTGGGGT -3') was used to detect specific nucleic acid of *H pylori*[19]. The template DNA (50 ng) and 20 pmol of each primer were added to a PCR mixture tube (Maxime PCR PreMix; iNtRON Biotechnology, Korea) containing 1 U of Taq DNA polymerase, 250 μmol/L each deoxynucleoside triphosphate, 50 mmol/L Tris-HCl (pH 8.3), 40 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and the gel loading dye. The volume was adjusted with distilled water to 20 μL. The

Table 1 Results of cultures and PCR for assessment of transmission of *H pylori* during pregnancy and at parturition day

Infection status	Evaluated time	Subject	Detection rate of *H pylori* (No. of positive/No. of animal)	
			Culture	PCR
Infected female	Pregnancy	Mothers	3/3	3/3
		Fetuses	0/23	0/23
	Delivery	Mothers	3/3	3/3
		Litters	0/21	0/21
	1-wk old	Mothers	3/3	3/3
		Fetuses	5/21	7/21
	3-wk old	Mothers	3/3	3/3
		Litters	11/23	16/23
Uninfected female	Pregnancy	Mothers	0/1	0/1
		Fetuses	0/7	0/7
	Delivery	Mothers	0/1	0/1
		Litters	0/9	0/9
	1-wk old	Mothers	0/1	0/1
		Litters	0/7	0/7
	3-wk old	Mothers	0/1	0/1
		Litters	0/7	0/7

reaction mixture was subjected to 30 amplification cycles (5 min at 95℃, 30 s at 94℃, 30 s at 52℃, 45 s at 72℃, and 5 min at 75℃) followed by a 5-min extension at 72℃ (model 9600 Thermocycler; Perkin-Elmer Cetus, USA). The PCR products were electrophoresed on a 1.2% agarose gel.

RESULTS

While culture of the bacterium is one of the gold standards in the diagnosis of *H pylori* infection, in the present study, we also used the PCR with the culture method to detect *H pylori*. The culture method was not considered to be ideal for determination of *H pylori* transmission because only small amounts of bacteria were suspected to colonize the stomach and the detection limit of the quantitative culture assay was 1×10^2 CFU/g gastric tissue[20]. The sensitivity of the PCR was 1 CFU/g feces and higher than those of other assays[19].Vertical transmission was examined at 2 wk after pregnancy and at parturition day (corresponding to the transplacental or intrauterine transmission during prenatal period and the delivery transmission during birth canal passage, respectively). Each stage group was composed of three pregnant females and their litters.

As the results of assessment performed at 2 wk after pregnancy, the mothers revealed *H pylori* infected status by culture assay and PCR (Table 1). However, their fetuses were not infected with *H pylori* (Figure 3). For the evaluation of delivery transmission during birth canal passage, all litters showed no transmission of *H pylori* (Figure 4) although the mothers were identified *H pylori* infected by culture assay and PCR (Table 1). However, their fetuses were not infected with *H pylori* (Table 1). *H pylori* was not detected in any litters and their mothers of the negative control group (Table 1).

Maternal transmission was examined at 1 wk and 3 wk postpartum (corresponding to the transitional milk and

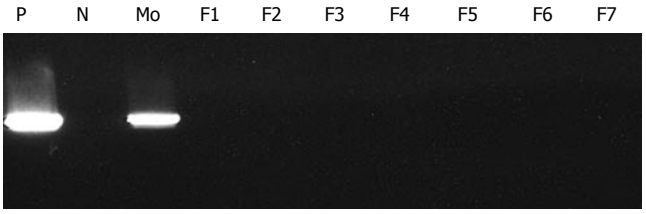

Figure 3 Amplification of Helicobacter DNAs. All fetuses showed no transmission of *H pylori* at 2 wk after pregnancy. P: Positive control; N: Negative control; Mo: Mother; F: Fetuses.

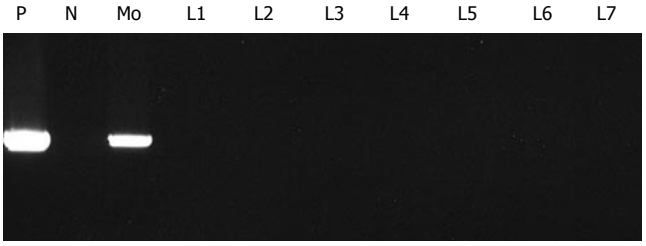

Figure 4 Amplification of specific nucleic acids for *H pylori*. All litters showed no transmission of *H pylori* at parturition day. P: Positive control; N: Negative control; Mo: Mother; L: Litters.

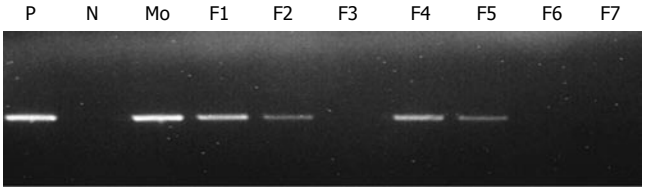

Figure 5 At 3 wk postpartum, some of litters delivered from infected mothers revealed positive reaction. P: Positive control; N: Negative control; Mo: Mother; L: Litters.

weaning stage, respectively). Each stage group was composed of three families. At 1 wk and 3 wk postpartum, the mothers revealed *H pylori* infected status by culture assay and PCR and some of their litters were infected with *H pylori* (Figure 5). The frequency of maternal transmission was increased during the nursing period. The transmission rate at 3 wk postpartum was significantly higher than at 1 wk postpartum (Table 1). *H pylori* was not detected in any litters of the negative control group.

DISCUSSION

Half of the world's population is estimated to be infected with *H pylori* and the infection is mainly acquired in early childhood but the exact routes of transmission remain elusive. Infected mothers are generally considered to be the main source of the pathogen[15,21,22]. The epidemiology of *H pylori* infection is variable, with prevalence being significantly higher and incident infection occurring earlier in developing countries compared with developed countries[17,23,24]. There is an obvious public health impact of *H pylori* infection and thus, to design targeted and cost-effective prevention strategies, elucidation of the mode of transmission for this bacterium is crucial[25]. It

is known that *H pylori* infection is typically acquired in early childhood and usually persists throughout life unless specific treatment is applied[12,14,26]. Definitive modes of transmission have not yet been characterized and the principal reservoir appears to be humans. Person-to-person transmission via fecal-oral, oral-oral and gastro-oral routes have been proposed[8-11]. Numerous studies also indicate low socioeconomic status, including domestic overcrowding in childhood, as major risk factors for higher infection prevalence rates[16,17,23,27,28]. Little is known about when and how often maternal transmission of *H pylori* occurs during the perinatal stage. In the present study, we examined these issues in an experimental murine model.

The results of the vertical-transmission experiment indicated that vertical transmission of *H pylori* did not occur at the pregnant and delivery stage. However, they revealed 33.3% and 69.6% at the lactating and weaning stage respectively. Recent epidemiological studies in humans suggest that acquisition of *H pylori* occur during childhood. For example, Rothenbacher *et al*[29] reported that *H pylori* acquisition seems to occurs mainly between the first and second year of life: that is, after the age of weaning. Our results are in agreement with this report. Also, Rothenbacher *et al*[22] reported that infected parents, especially infected mothers, play a key role in the transmission of *H pylori* within families. Maternal contact behaviour during the breastfeeding period may be responsible for the high frequency of maternal transmission[30]. Our results also showed that the maternal-transmission of *H pylori* was not observed during pregnancy and delivery stage, but detected at lactating and weaning stage. On the basis of these findings, vertical infection during pregnancy or at delivery is unlikely as a route of mother-to-child *H pylori* infection. We suggested that *H pylori* infection by the transplacental route during pregnancy does not occur and that *H pylori* transmission by discharges of the uterine or vagina, obstetric delivery tract, during parturition does not be occur. *H pylori* might be acquired through breast-feeding, contaminated saliva and fecal-oral transmission during co-habitation.

In conclusion, the present study provides new and important information on maternal transmission of *H pylori*. This study implied that maternal transmission of *H pylori* might be developed during latency or a later postpartum stage. Data from human children are limited, because most *H pylori*-infected children have no symptoms and it is difficult for a paediatrician to examine such asymptomatic children invasively. In the present study, we examined these issues in an experimental murine model, the Mongolian gerbil model that has been reported as an optimal laboratory animal model to study *H pylori in vivo*[18]. We analysed the stomachs of many infant Mongolian gerbils directly and we believe that it is meaningful to use our results to speculate when *H pylori* infection occurs in human children. The acquisition of *H pylori* infection during childhood seems to be a critical risk factor for the later development of gastric cancer. The prevention of transmission of *H pylori* during childhood could provide an effective strategy to decrease *H pylori* infection and gastric cancer.

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REFERENCES

1 Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983; **1**: 1273-1275
2 **Matysiak-Budnik T**, Mégraud F. *Helicobacter pylori* infection and gastric cancer. *Eur J Cancer* 2006; **42**: 708-716
3 **NIH Consensus Conference**. *Helicobacter pylori* in peptic ulcer disease. NIH Consensus Development Panel on *Helicobacter pylori* in Peptic Ulcer Disease. *JAMA* 1994; **272**: 65-69
4 **Peek RM Jr**, Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* 2002; **2**: 28-37
5 Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *IARC Monogr Eval Carcinog Risks Hum* 1994; **61**: 1-241
6 **Honda S**, Fujioka T, Tokieda M, Satoh R, Nishizono A, Nasu M. Development of *Helicobacter pylori*-induced gastric carcinoma in Mongolian gerbils. *Cancer Res* 1998; **58**: 4255-4259
7 **Watanabe T**, Tada M, Nagai H, Sasaki S, Nakao M. *Helicobacter pylori* infection induces gastric cancer in mongolian gerbils. *Gastroenterology* 1998; **115**: 642-648
8 **Kim N**. [Epidemiology and transmission route of *Helicobacter pylori* infection]. *Korean J Gastroenterol* 2005; **46**: 153-158
9 **Kikuchi S**, Dore MP. Epidemiology of *Helicobacter pylori* Infection. *Helicobacter* 2005; **10** Suppl 1: 1-4
10 **Mladenova I**, Durazzo M, Pellicano R. Transmission of *Helicobacter pylori*: are there evidences for a fecal-oral route? *Minerva Med* 2006; **97**: 15-18
11 **Torres J**, Pérez-Pérez G, Goodman KJ, Atherton JC, Gold BD, Harris PR, la Garza AM, Guarner J, Muñoz O. A comprehensive review of the natural history of *Helicobacter pylori* infection in children. *Arch Med Res* 2000; **31**: 431-469
12 **Crone J**, Gold BD. *Helicobacter pylori* infection in pediatrics. *Helicobacter* 2004; **9** Suppl 1: 49-56
13 **Rothenbacher D**, Bode G, Berg G, Knayer U, Gonser T, Adler G, Brenner H. *Helicobacter pylori* among preschool children and their parents: evidence of parent-child transmission. *J Infect Dis* 1999; **179**: 398-402
14 **Rowland M**, Daly L, Vaughan M, Higgins A, Bourke B, Drumm B. Age-specific incidence of *Helicobacter pylori*. *Gastroenterology* 2006; **130**: 65-72; quiz 211
15 **Weyermann M**, Adler G, Brenner H, Rothenbacher D. The mother as source of *Helicobacter pylori* infection. *Epidemiology* 2006; **17**: 332-334
16 **Bardhan PK**. Epidemiological features of *Helicobacter pylori* infection in developing countries. *Clin Infect Dis* 1997; **25**: 973-978
17 **Frenck RW Jr**, Clemens J. Helicobacter in the developing world. *Microbes Infect* 2003; **5**: 705-713
18 **Hirayama F**, Takagi S, Yokoyama Y, Iwao E, Ikeda Y. Establishment of gastric *Helicobacter pylori* infection in Mongolian gerbils. *J Gastroenterol* 1996; **31** Suppl 9: 24-28
19 **Kim SH**, Kim O. Application of Consensus Polymerase Chain Reaction for Monitoring of *Helicobacter* Species. *Kor J Lab Anim Sci* 2004; **20**: 316-320
20 **Minoura T**, Kato S, Otsu S, Fujioka T, Iinuma K, Nishizono A. Childhood *Helicobacter pylori* infection in a murine model: maternal transmission and eradication by systemic immunization using bacterial antigen-aluminium hydroxide. *Clin Exp Immunol* 2003; **134**: 32-37
21 **Escobar ML**, Kawakami E. Evidence of mother-child transmission of *Helicobacter pylori* infection. *Arq Gastroenterol* 2004; **41**: 239-244

22 **Rothenbacher D**, Winkler M, Gonser T, Adler G, Brenner H. Role of infected parents in transmission of *helicobacter pylori* to their children. *Pediatr Infect Dis J* 2002; **21**: 674-679
23 **Ahuja V**, Sharma MP. High recurrence rate of *Helicobacter pylori* infection in developing countries. *Gastroenterology* 2002; **123**: 653-654
24 **Graham DY**, Adam E, Reddy GT, Agarwal JP, Agarwal R, Evans DJ, Malaty HM, Evans DG. Seroepidemiology of *Helicobacter pylori* infection in India. Comparison of developing and developed countries. *Dig Dis Sci* 1991; **36**: 1084-1088
25 **Fendrick AM**, Chernew ME, Hirth RA, Bloom BS, Bandekar RR, Scheiman JM. Clinical and economic effects of population-based *Helicobacter pylori* screening to prevent gastric cancer. *Arch Intern Med* 1999; **159**: 142-148
26 **Jones NL**, Sherman PM. *Helicobacter pylori* infection in children. *Curr Opin Pediatr* 1998; **10**: 19-23
27 **Fiedorek SC**, Malaty HM, Evans DL, Pumphrey CL, Casteel HB, Evans DJ Jr, Graham DY. Factors influencing the epidemiology of *Helicobacter pylori* infection in children. *Pediatrics* 1991; **88**: 578-582
28 **Mendall MA**, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Northfield TC. Childhood living conditions and *Helicobacter pylori* seropositivity in adult life. *Lancet* 1992; **339**: 896-897
29 **Rothenbacher D**, Inceoglu J, Bode G, Brenner H. Acquisition of *Helicobacter pylori* infection in a high-risk population occurs within the first 2 years of life. *J Pediatr* 2000; **136**: 744-748
30 **Kurosawa M**, Kikuchi S, Inaba Y, Ishibashi T, Kobayashi F. *Helicobacter pylori* infection among Japanese children. *J Gastroenterol Hepatol* 2000; **15**: 1382-1385

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BASIC RESEARCH

Mechanical behavior of colonic anastomosis in experimental settings as a measure of wound repair and tissue integrity

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Abstract

AIM: To determine the mechanical properties of anastomotic colonic tissue in experimental settings and therefore give a measure of wound healing.

METHODS: Thirty-six male Wistar rats were used as experimental models of anastomotic tissue integrity. On the 5th post-operative day, the tensile strength was measured by application of an axial force, providing a quantitative measure of anastomotic dehiscence and leakage.

RESULTS: Diagrams of the load as a function of the time [P = P (t)] and of the displacement also as a function of time [Δs = Δs (t)] were recorded for each test, permitting the design of the load versus the displacement diagram and thus providing significant data about the critical values of anastomotic failure. Quantitative data were obtained concerning the anastomotic strength of both control specimens (healthy rats), as well as specimens from non-healthy rats for comparison.

CONCLUSION: This experimental model provides an excellent method of measuring anastomotic strength. Despite the relative small number of specimens used, this method provides an accurate way of measuring wound repair. More experimental measurements need to be performed to correlate emerging tensile strength values to anastomotic failure.

Key words: Mechanical behavior; Failure load; Colon; Anastomosis; Wound healing

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INTRODUCTION

Investigating wound healing and attempting to improve its outcome necessitates process quantification[1]. Parameters for anastomotic repair and adhesion formation[2] may be mechanical, biochemical, or histological. Histology is not a primary tool for quantification when comparing various series of experimental anastomoses. Certainly it is very useful to describe the course and eventual result of the healing sequence at the tissue level. Also, the successive infiltration of various cells into the wound area may be followed, and obvious differences between anastomoses (e.g., ileal and colonic) will certainly be demonstrated this way. However, the measurement of choice to evaluate anastomotic repair and the effects of variations in surgical techniques, administration of drugs, or of any other modification to establish procedures, will mostly be either mechanical or biochemical or both.

The developing mechanical strength is, without doubt, a meaningful parameter to follow while investigating anastomotic healing. For this purpose, two fundamentally different approaches can be chosen. First, one can choose bursting strength, which is expressed either as bursting pressure or bursting wall tension, which is the measure of the resistance of the intestinal wall to increasing intraluminal pressure. Second, one can choose breaking strength, which reflects the resistance of the intestinal wall to forces exerted in a longitudinal direction[3-5].

While both of these methods used to evaluate anastomotic healing have been investigated in the international literature, no paper has so far described

in detail the process itself, analyzing its advantages, disadvantages and parameters taken into consideration, therefore establishing the need for an in-depth presentation of the mechanical apparatus used and presenting not only the theoretical background behind the measurements but also the technical difficulties that arise.

MATERIALS AND METHODS

Thirty-six male Wistar rats weighing 300-350 g were used, and were housed two per cage. They were fed a standard diet and water *ad libitum*. All experiments were approved by the Athens Prefecture, Directorate of Veterinary Services (License No. K/355/27-1-2005), according to the Presidential Decree No. 160/1991 (Governmental Gazette A' 64), with which Greece has conformed to the 86/609/EEC directive. Laparotomy[6] was performed through a midline 2 cm incision under anesthesia induced by ketamine (80 mg/kg) and xylazine (3 mg/kg). A colonic segment, 1 cm in length, 5 cm distal to the ileocecal junction was transected and the colon was re-anastomosed end-to-end using 5-0 Vicryl (Ethicon) sutures in single-layer interrupted fashion[7]. About 10 sutures were placed for each anastomosis to secure an inverted anastomosis without mucosal protrusion, which is regarded as a major cause of perianastomotic adhesions. The abdominal muscle wall was then closed with 5-0 Vicryl (Ethicon) sutures, followed by skin closure with 4-0 Silk (Medipac) sutures.

To obtain the test specimen, the rats were sacrificed with an overdose of ether, on the 5th post-operative day. The previous abdominal incision was reopened, and the anastomotic site identified and inspected for possible adhesions and leakage. An 8 cm segment of the colon with the anastomosis in the middle was resected. Care was taken not to detach adhesions from the anastomosis, but to dissect the surrounding tissues. The resected specimen was gently irrigated with saline to remove feces and was mounted on a table.

The basic purpose of the present experimental protocol is the determination of the mechanical behavior of intestinal anastomoses and more specifically the response to tensile loading and the determination of the respective tensile strength. One can define the ratio of the applied force at the moment of failure, F_{cr}, over the surface, A, upon which the force acts normally[8], as tensile strength (Figure 1). The ratio of the force over the respective area is known in engineering science as stress and therefore the tensile strength is the respective tensile stress at the moment of failure.

In the international scientific literature the mechanical behavior under tension, of specimens like the ones of the present protocol, has been studied in two ways: (1) By applying an internal hydraulic pressure, p,[9-12]. In this case (and for points relatively far from the borders of the specimens) a stress state equivalent to the so-called biaxial tension appears on the surface of the concave cylindrical specimen. Assuming that the thickness of the specimens is much smaller in comparison to its diameter, the principal tensile stresses at the moment of failure are given by the

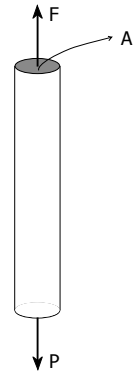

Figure 1 Typical forces applied on a standard specimen.

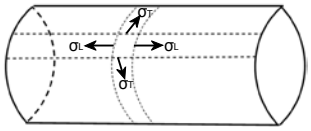

Figure 2 The application of hydraulic pressure creating a two-dimensional stress field.

relations (Figure 2)[13]:

$$\sigma_L = \frac{p_{cr} r}{2t}, \quad \sigma_T = \frac{p_{cr} r}{t}$$

where p_{cr} is the value of the hydraulic pressure at the moment of the first failure, r the radius of the intestine and t its wall thickness. The stress system is characterized as principal since no shear stresses can be generated by a pure hydraulic pressure.

(2) By applying directly an axial force, F,[14,15]. In this case the critical value of the tensile strength is expressed as:

$$\sigma_{cr} = \frac{F_{cr}}{2\pi rt}$$

The procedures described above present both advantages and disadvantages. More specifically, the application of hydraulic pressure, although relatively easily realizable in the laboratory, creates a two-dimensional stress field, as shown in Figure 2. Therefore the conclusions drawn are not directly comparable to the respective ones of the experiments with uniaxial loading. Especially in the case of anisotropic materials (like the ones of the present study), it is impossible to define which one of the two stresses is responsible for the failure and therefore it is not possible to determine the critical value, since the direction at which failure will appear is not *a priori* known.

On the contrary, the application of an axial force is especially difficult from a practical point of view (as it will be seen in the next paragraph), but the results obtained are directly usable without reductions and additional assumptions.

Experimental difficulties of the direct tension experiment

In the present study the second procedure (direct tension) was adopted. The most important difficulties stated above

are summarized as following: (1) The nature of the materials under study renders the gripping of the specimens, with the aid of conventional friction grips through compression loads, extremely difficult. In fact, since it is impossible to form "gripping heads" to the specimens ("dog-bone" specimens), it is given that the failure will appear in the portion of the specimen which is inside the grips or in their immediate vicinity. However, in this area the stress field is strongly triaxial and therefore the results obtained are invalid and should be rejected. On the other hand, the limited chances to obtain long specimens in combination with the low friction coefficient between the external surfaces of the specimens (intestines) do not allow the use of pulley-shaped grips, in which the holding force emanates from the friction of a number of successive layers of the material rolled around the periphery of the pulley. (2) The extremely low force which is necessary for the fracture of even intact and healthy specimens, which according to international literature is estimated at the value of a few tenths of Newton[14], renders the conventional arrangements of applying axial tension practically useless. (3) The nature of the specimens under study, which are twisted and bended around different axes, due to adhesion formation around the anastomotic area, renders the measurements of length changes and therefore of reduced deformations (strains, ε) almost impossible. (4) Finally, the nature of the intestines once again, which under torsion are "self-configured" into the form of plane plates, results in an interaction between the walls of the specimens, making difficult the reduction of the external loads into stresses (σ). Another factor making the situation more difficult is the non-constant thickness of the specimens throughout their length and their perimeter, which does not allow us to calculate the effective area of the loaded intestine.

In order to confront these difficulties in the present experimental study, the following procedures were adopted.

Gripping the specimens

A specific gripping system was designed, consisting of a pair of light metallic pins of cylindrical cross-section of diameter equal to 5 mm, with rounded head which permits easy entrance of the intestine in the pin, without injuring the specimen walls, reducing thus the time required for the in-situ preparation of the specimens (Figure 3). The pins are grooved at their mid-length and a suture which holds the specimens in place is rolled up in this groove. The upper part of the pins is drilled through the thickness and the specimen is suspended through this hole from the upper plate of the loading frame. At the same time, the second pin is fixed to the immobile plate of the frame.

The suspension and fixing of the pins is achieved with the aid of circular rings. In this way the maximum possible number of degrees of freedom is given to the specimen making possible the self-alignment and the "untwisting" of the intestine during tension without external limitations and therefore without, as much as possible, the development of parasitic tensions and disfigurations.

Despite the low total weight of the specimens gripping system (about 0.12 N), it was deemed appropriate to add the weight of the lower half, which is suspended and therefore sustained by the specimen, at the value of the final failure load, taken into consideration that these values are relatively comparable (the mean value of the failure load, as it was obtained from a series of preliminary experiments is equal to about ranges between 1.00 N and 2.20 N).

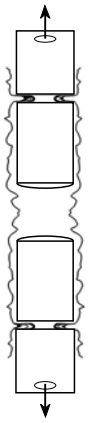

Figure 3 The gripping system consisting of a pair of light metallic pins. The grooves at which the intestine is gripped are indicated by the arrows.

The load application system

After the rejection of loading through the application of dead weights (water or lead grains), due to the induction of vibrations and oscillations, a special load cell of capacity of 5 N and sensitivity of 10^{-3} N was used attached in a stiff electrical loading frame (Instron). This frame was selected, apart from its robustness, because it provides the ability of choosing the load application speed between wide ranges (from 0.5 mm/min to 500 mm/min). This characteristic of the frame is very important in case biological materials are to be studied, since their mechanical behavior exhibits viscoelastic nature, which is strongly dependent on the strain rate induced ($d\varepsilon/dt$).

In the first phase of the experimental project an especially low tension speed (1 mm/min) was selected, and therefore the loading can be considered as static or at least quasi-static. As a result the overall duration of each test usually exceeds 10 min. It is planned, in a second phase, to study the effect of loading rate by employing dynamic or quasi-dynamic protocols.

Calibration of the apparatus

Before starting the main series of experiments, a number of preliminary tests were carried out, in order to define the range of the expected values of both the failure load and the elongation of typical specimens, and to calibrate the apparatus in the specific range of values.

The calibration of the loads was achieved with the safest method of the suspension of standardized (certified) weights from the load cell. Both the absolute reading values of the load cell as well as their linearity at the range of the expected loads were checked. The deviations detected for the absolute values of the loads did not exceed in any case the limit of 0.2% set by the "Quality Assurance System" of the Laboratory of Testing and

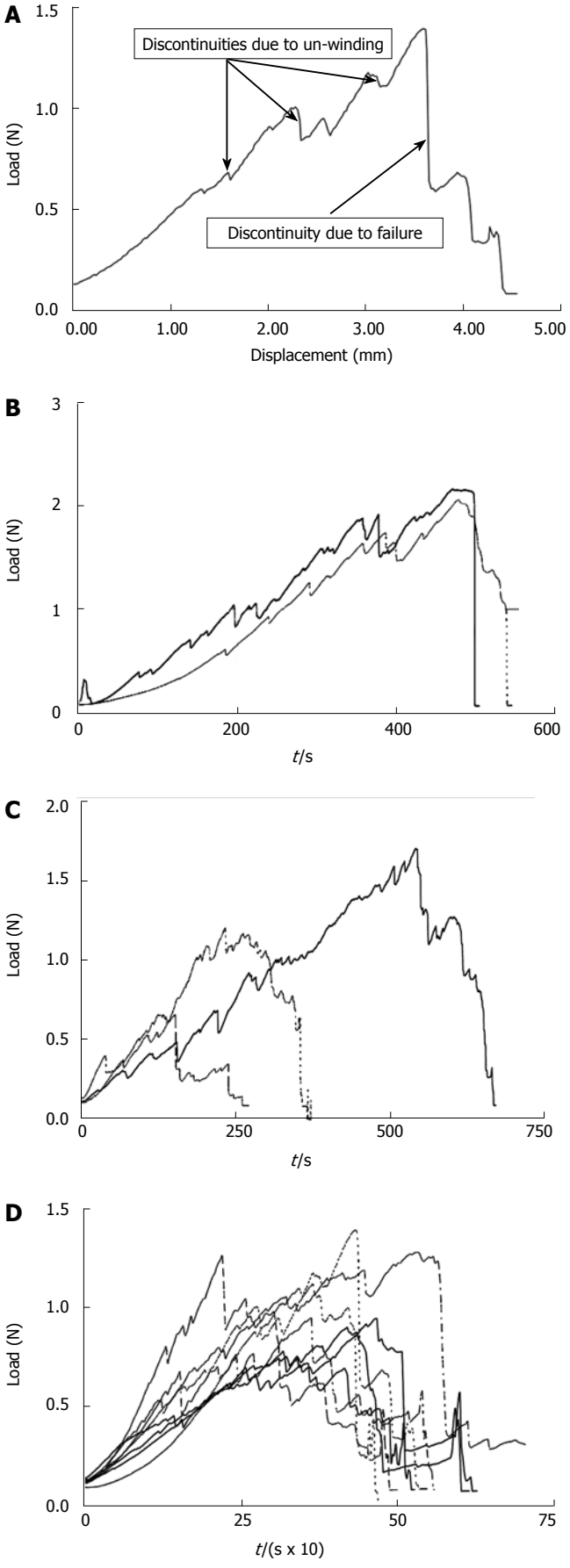

Figure 4 Load versus time and displacement diagrams for characteristic tests. **A**: Load versus displacement for a typical test of the preliminary series; **B**: Load versus time using intact specimens from healthy rats; **C**: Load versus time using specimens from healthy rats after colonic anastomosis; **D**: Load versus time using specimens from non-healthy rats after colonic anastomosis.

Materials of the National Technical University of Athens (NTUA/LTM), as it is described in the respective "Quality Assurance Manual" according to ISO9000/2000 system.

On the other hand, the linearity of the values of the loading cell in relation to the respective ones of the standard weights exceeded 99.8% for the whole range of interest, as it was concluded from a linear interpolation in the experimental data, using the least square method.

The calibration of the readings of the load frame for the displacements was achieved with the aid of three LVDT's (Linear Voltage Displacement Transducers), which have been verified with a standard micrometric vernier of an accuracy of 1 μm. Apart from the absolute values of the displacements, the parallel of the motion of the loading frame was also checked. The deviations detected did not exceed in any case the limits set by "Quality Assurance System" of the NTUA/LTM. Finally, the time recording device of the data acquisition and storage system was also calibrated with the aid of a prototype chronometer. The deviations were not measurable.

Data acquisition and storage system

The data to be recorded during the experiments include the values of the load as a function of the time [P = P (t)] and the values of the displacement of the moving plate of the loading frame also as a function of time [Δs = Δs (t)]. The data acquisition system includes a special multi channel "bridge" (National Instruments, type SCXI-1000), with the ability of adjusting the sampling rate. The system includes, also, a personal computer with suitable commercial software (LabVIEW-8). From the functions F = F (t) and Δs = Δs (t) recorded, one can eliminate the time obtaining the function of the applied force as a function of the displacement induced and therefore as a function of the elongation of the intestine, i.e. F = F (Δs).

After the preliminary experiments, it was deemed appropriate to add to the data acquisition system a video device, in order to monitor the specimen during the experiment in a mode synchronous to the recording of the values of the load and the displacement. This was considered necessary, since the records of the load versus presented oscillations, due to two different reasons: (1) The "un-twisting" of the twisted parts or the "un-folding" of the folded parts of the intestine, which lead to a sudden length increase of the specimen, and therefore to instantaneous unloading, that is to a fall in the recorded load, as it is shown characteristically in the diagram of Figure 4A. (2) Local failures of parts of the specimen and especially in the case of anastomosed intestines failure of the anastomotic area itself or of directly neighboring areas, due to the tearing of the material from the anastomotic suture.

Since one cannot distinguish between these two discontinuities of the F = F (t) diagram, the synchronous video-recording of the experiment was considered necessary. In this way it is possible to locate the discontinuities of the diagram due to the "un-twisting" or to the "un-folding" of the specimen, until the discontinuity due to the anastomotic failure or failure of its immediate neighboring area. Therefore the loading corresponding to this discontinuity can be safely considered as the crucial anastomotic failure load.

RESULTS

Three different classes of specimens were tested using the system described in the previous paragraphs.

The first one included a number of "intact" specimens, i.e. specimens from healthy rats without anastomoses. The results of these tests are to be used as a measure that will permit the characterization of the quality of the anastomosis, at least from the point of view of mechanical strength. Two characteristic examples of these tests are shown in Figure 4B. The data obtained from these tests for the failure force exhibited very small scattering (as it was perhaps expected) and the average value was of the order of:

$$F_{cr}^{intact} = 2.09 \text{ N} \pm 0.6 \text{ N}$$

Taking into account that the thickness of the wall of the intestine of the rats after the 8th week of their life is stabilized to about 1.1 mm while its perimeter varies in the range 9-12 mm[16] , it is concluded that the tensile failure strength of the "intact" specimens ranges between:

$$160 \text{ kPa} \leqslant \sigma_{fail}^{intact} \leqslant 210 \text{ kPa}$$

The second class of experiments included the control tests, namely it was carried out using specimens obtained from healthy rats but after having been subjected to colon anastomoses. The scattering of this series of tests was obviously higher compared to that of the "intact" specimens and it was considered necessary to study the acceptability of the results based on statistical experiments. The Chauvenet criterion was adopted and a number of tests were excluded from the analysis. In Figure 4C the results of three tests of this series are shown, corresponding to the ones with the lowest and highest acceptable failure forces and to a third one with failure force almost equal to the average value. The average value for the failure force was determined equal to:

$$F_{fail}^{control} = 1.35 \text{ N} \pm 0.42 \text{ N}$$

Similarly the failure strength ranges between:

$$100 \text{ kPa} \leqslant \sigma_{fail}^{control} \leqslant 135 \text{ kPa}$$

It can be concluded that the present procedure for the anastomotic operation results in a decrease of the mechanical strength of the colonic segments under study of the order of only 35% in comparison to the intact specimens.

As a final step, a third series of tests was carried out with specimens obtained from non-healthy rats after having been subjected to colon anastomotic surgery. It was strange to observe that the scattering of the results was rather lower in this case and the application of the Chauvenet criterion yielded the exemption of only one test. A number of characteristic tests of this class experiments is shown in Figure 4D. The average value for the failure force, for this series of tests was determined equal to:

$$F_{fail}^{non\text{-}healthy} = 1.09 \text{ N} \pm 0.19 \text{ N}$$

In this case failure strength ranges between:

$$82 \text{ kPa} \leqslant \sigma_{fail}^{non\text{-}healthy} \leqslant 110 \text{ kPa}$$

The decrease of the mechanical strength compared to the intact specimens is of the order of about 50%, while if the comparison is carried out on the basis of the results of the control tests, is of the order of about 19%.

DISCUSSION

Wound leakage, the major concern for every surgeon performing intestinal anastomosis, is considered a multifactorial process, upon which many factors act, accelerating or inhibiting its metabolic pathway[17,18]. Numerous clinical entities and metabolic abnormalities can alter the course of tissue repair. Amongst them diabetes mellitus, hypothyroidism, immunocompetence, infection and other diseases are proven to be detrimental to anastomotic healing, while other factors like the surgical technique, advanced age, malnutrition, obesity, inadequate perfusion and/or oxygenation are considered risk factors for impaired wound healing[19-22].

Taking the 5th post-operative day as a crucial time point upon which anastomotic failure is mostly recognized in clinical practice, the authors tried to give a measure of the anastomotic strength by taking advantage of its mechanical behavior. While both bursting pressure and tensile strength are used to describe the mechanical properties of viscoelastic materials like the ones under study, the authors preferred to evaluate the second and correlate it to the healing of colonic anastomosis. This is because tensile strength appears to be a better standard to evaluate the biological aspects of healing. Tensile strength is an important determinant of anastomotic strength, in contrast to the bursting pressure, which can evaluate the overall anastomotic integrity, but may reflect healing less accurately.

The authors in this paper described not only a system for gripping the specimens, the load application, the data acquisition and storage system, but also a detailed view of the theoretical background behind the forces applied in the tissues under study, as well as the experimental difficulties of the direct tension experiment.

The values of the load as a function of the time [P = P (t)] and the values of the displacement of the moving plate of the loading frame also as a function of time [$\Delta s = \Delta s$ (t)] were recorded, giving the load versus the displacement curve for each measurement and therefore providing the recorded discontinuities due to the anastomotic failure.

While the tests performed were used only for a preliminary series of measurements, since the number of specimens was relatively small, significant conclusions can be made regarding wound strength and tissue regeneration. The decrease of the axial force required causing mechanical failure from 2.09 N in case of the "intact" specimens to about 1.35 N for the control tests and to 1.09 N for the specimens from non-healthy rats is an excellent index of the quality of the anastomotic operation. Of course, a larger number of measurements need to be carried out, so as to provide a more rigid approach to tissue leakage, its quantative expression through the tensile strength experiments, and its clinical correlations with pathological entities that delay wound healing or with factors that promote anastomotic integrity and repair.

REFERENCES

1 **Zografos GC**, Martis K, Morris DL. Laser Doppler flowmetry in evaluation of cutaneous wound blood flow using various

suturing techniques. *Ann Surg* 1992; **215**: 266-268
2 **Zografos GC**, Simeonidis KM, Parasi AS, Messaris EG, Menenakos EE, Dontas IA, Marti KC, Androulakis GA. Adhesion formation: intraperitoneal catheters in surgical practice. *J Invest Surg* 2002; **15**: 37-43
3 **Hendriks T**, Mastboom WJ. Healing of experimental intestinal anastomoses. Parameters for repair. *Dis Colon Rectum* 1990; **33**: 891-901
4 **Ikeuchi D**, Onodera H, Aung T, Kan S, Kawamoto K, Imamura M, Maetani S. Correlation of tensile strength with bursting pressure in the evaluation of intestinal anastomosis. *Dig Surg* 1999; **16**: 478-485
5 **Koruda MJ**, Rolandelli RH. Experimental studies on the healing of colonic anastomoses. *J Surg Res* 1990; **48**: 504-515
6 **Komarek V**. Gross anatomy. In: Krinke GJ. The laboratory rat. London: Academic Press, 2000: 253-264
7 **Verhofstad MH**, Bisseling TM, Haans EM, Hendriks T. Collagen synthesis in rat skin and ileum fibroblasts is affected differently by diabetes-related factors. *Int J Exp Pathol* 1998; **79**: 321-328
8 **Popov EP**. Stress - axial loads - safety. In: Popov EP. Engineering Mechanics of Solids. New Jersey: Prentice Hall, 1990: 1-30
9 **Wheeless CR Jr**, Zanagnolo V, Bowers D, Brenner MJ, Lilley R. The effect of growth hormone on the bursting strength of ileal anastomotic segments in radiation-injured rat bowel. *Gynecol Oncol* 1998; **70**: 121-122
10 **Sweeney T**, Rayan S, Warren H, Rattner D. Intestinal anastomoses detected with a photopolymerized hydrogel. *Surgery* 2002; **131**: 185-189
11 **Stoop MJ**, Dirksen R, Hendriks T. Advanced age alone does not suppress anastomotic healing in the intestine. *Surgery* 1996; **119**: 15-19
12 **Waninger J**, Kauffmann GW, Shah IA, Farthmann EH. Influence of the distance between interrupted sutures and the tension of sutures on the healing of experimental colonic anastomoses. *Am J Surg* 1992; **163**: 319-323
13 **Ugural AC**. Concept of stress. In: Ugural AC. Mechanics of Materials. New York: McGraw Hill, 1991: 11-28
14 **Jönsson K**, Jiborn H, Zederfeldt B. Breaking strength of small intestinal anastomoses. *Am J Surg* 1983; **145**: 800-803
15 **Tani T**, Tsutamoto Y, Eguchi Y, Araki H, Ebira Y, Ameno H, Fujino M, Oka H, Kodama M. Protease inhibitor reduces loss of tensile strength in rat anastomosis with peritonitis. *J Surg Res* 2000; **88**: 135-141
16 **Lu X**, Zhao J, Gregersen H. Small intestinal morphometric and biomechanical changes during physiological growth in rats. *J Biomech* 2005; **38**: 417-426
17 **Stadelmann WK**, Digenis AG, Tobin GR. Impediments to wound healing. *Am J Surg* 1998; **176**: 39S-47S
18 **Norris SO**, Provo B, Stotts NA. Physiology of wound healing and risk factors that impede the healing process. *AACN Clin Issues Crit Care Nurs* 1990; **1**: 545-552
19 **Ekmektzoglou KA**, Zografos GC. A concomitant review of the effects of diabetes mellitus and hypothyroidism in wound healing. *World J Gastroenterol* 2006; **12**: 2721-2729
20 **Silhi N**. Diabetes and wound healing. *J Wound Care* 1998; **7**: 47-51
21 **Mäkelä JT**, Kiviniemi H, Laitinen S. Risk factors for anastomotic leakage after left-sided colorectal resection with rectal anastomosis. *Dis Colon Rectum* 2003; **46**: 653-660
22 **Natori J**, Shimizu K, Nagahama M, Tanaka S. The influence of hypothyroidism on wound healing. An experimental study. *Nihon Ika Daigaku Zasshi* 1999; **66**: 176-180

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BASIC RESEARCH

Localization of ANP-synthesizing cells in rat stomach

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Abstract

AIM: To study the morphological positive expression of antrial natriuretic peptide (ANP)-synthesizing cells and ultrastructural localization and the relationship between ANP-synthesizing cells and microvessel density in the stomach of rats and to analyze the distribution of the three histologically distinct regions of ANP-synthesizing cells.

METHODS: Using immunohistochemical techniques, we studied positive expression of ANP-synthesizing cells in rat stomach. A postembedding immunogold microscopy technique was used for ultrastructural localization of ANP-synthesizing cells. Microvessel density in the rat stomach was estimated using tannic acid-ferric chloride (TAFC) method staining. Distribution of ANP-synthesizing cells were studied in different regions of rat stomach histochemically.

RESULTS: Positive expression of ANP-synthesizing cells were localized in the gastric mucosa of rats. Localization of ANP-synthesizing cells identified them to be enterochrochromaffin cells (EC) by using a postembedding immunogold electron microscopy technique. EC cells were in the basal third of the cardiac mucosa region. ANP-synthesizing cells existed in different regions of rat stomach and its density was largest in the gastric cardiac region, and the distribution order of ANP-synthesizing cells in density was cardiac region, pyloric region and fundic region in mucosa layer. We have also found a close relationship between ANP-synthesizing cells and microvessel density in gastric mucosa of rats using TAFC staining.

CONCLUSION: ANP-synthesizing cells are expressed in the gastric mucosa. EC synthesize ANP. There is a close relationship between ANP-synthesizing cells and microvessel density in gastric mucosa of rats.The distribution density of ANP-synthesizing cells is largest in the gastric cardiac region.

Key words: Antrial natriuretic peptide-synthesizing cells; Microvessel density; Close relationship; Gastric cardiac region

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INTRODUCTION

Since antrial natriuretic peptide (ANP) was isolated from atrium by de Bold *et al* in 1981[1-3], brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis (DNP), micrurus natriuretic peptide (MNP), and ventricular natriuretic peptide (VNP) have been found in succession. They distribute not only in the heart but all over the body[4-10]. ANP regulates a variety of physiological functions, including natriuresis, diuresis and vasodilation. Three types of natriuretic peptide receptors for ANP, BNP and CNP have been identified, for example, natriuretic peptide receptor type A (NPR-A), type B (NPR-B) and type C (NPR-C). NPR-A has guanylate cyclase activity and mediates the biological functions of ANP through the synthesis of cGMP[2]. NPR-A preferentially binds ANP and BNP, but has a low affinity for CNP. Although ANP is synthesized primarily in the heart as a cardiac hormone, in fact ANP and its receptor are expressed in numerous extracardiac tissues, e.g., lung, thymus, gastrointestinal tract, suggesting a possible role as a regional regulator acting as an autocrine and/or paracrine regulatory peptide[11,12]. Our previous study indicated that NPR existed in different regions of gastric mucosa and its density was the largest in rat gastric antrum, and NPR significantly inhibited spontaneous contraction of gastric smooth muscles in rats, guinea-pigs and humans[13-16]. However, the distribution of ANP-synthesizing cells and the relationship between the distribution of ANP-synthesizing cells and microvessel density in gastric tissues have not been identified. It is also not clear about the ultrastructural localization of ANP-synthesizing cells in rat stomach. Therefore, in the present

study, the morphological distribution and ultrastructural localization of ANP-synthesizing cells were identified under postembedding immunoelectron microscopy. The relationship between distribution of ANP-synthesizing cells and microvessel density was investigated using histochemical techniques in rat stomach.

MATERIALS AND METHODS

Animals

Wistar rats (obtained from the Experimental Animal Center of Yanbian University College of Medicine) of either sex weighing 300-350 g were anaesthetized by a lethal dose of abdominal cavity injection of pentobarbital sodium (30 mg/kg), and the abdomen of each rat was opened along the midline. Because the rat stomach demonstrates significant regional differences in structure, we separated the stomach into three regions: the fundic or fundus, the cardiac, and the pyloric region or antrum (Figure 1).

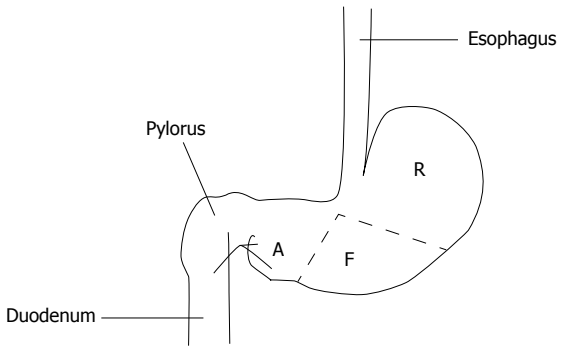


Figure 1 Different histological regions of rat stomach. R: Cardic region or cardia; F: Fundic region or fundus; A: Pyloric region or antrum.

Immunohistochemistry

Freshly excised atria and stomach were fixed in 40 g/L foermaldehyde fixative and embedded in paraffin. Sections (5 μm) were deparaffinated, rehydrated and incubated with 3 mL/L hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase activity. After washing twice with phosphate-buffered saline (PBS) for 5 min, tissue sections were incubated at 37℃ for 20 min with blocking solution. Sections were incubated at 37℃ for 2 h with primary antibody: rabbie anti-rat ANP (sc-20158, Santa Cruz Biotechnology, Inc) 1:100. After washing twice with PBS (0.01 mol/L, pH7.4) for 10 min, tissue sections were incubated at 37℃ for 30 min with biotin-anti-rabbit IgG. After washing two times in PBS for 5 min, the sections were incubated with streptavidin-HRP for 30 min. Then the sections were washed two times in PBS for 5 min, and they were incubated with metal-enhanced 3,3-diaminobenzidene solution for 15 min, then they were washed two times in distilled water and counterstained with hematoxylin. Negative control sections were incubated with normal rabbit serum instead of primary antibody. The positive staining for ANP-synthesizing cells was expressed as red brown granules, which were mainly located in cell cytoplasm under microscopy. At least 5 high-power (× 400 field) fields were chosen randomly for cell counting. The ratio of the positive distribution of ANP-synthesizing cells was calculated by dividing the number of positive cells over the total number of cells, and was expressed as percentage, counted , analyzed under the CMIAS image analysis system (Beihang, China), and photomicrographed (Olympus PM-10AD).

Immunoelectron microscopy

Freshly excised stomach mucosal and atrial myocyte blocks of 1 mm^3 were fixed in 100 mL/L paraform fixative, then dehydarated and embedded in Epon-812 resin. Sections (70 nm) were incubated with 100 mL/L hydrogen peroxide in methanol for 30 min at room temperature. After washing twice with distilled water for 10 min, tissue sections were incubated at room temperature for 1h with blocking solution, then tissue sections were incubated at 4℃ for 36 h with primary antibody: rabbit anti-rat ANP (sc-20158, Santa Cruz Biotechnology, Inc) 1:90. Negative control sections were incubated with normal rabbit serum instead of primary antibody. After washing twice with PBS for 5 min, tissue sections were incubated at room temperature for 1h with protein A-10 nm colloidal gold labeled (Product Number P 6730, Sigma). After washing twice with distilled water for 5 min, sections were stained with urenyl acetate and lead citrate each for 5 min and were observed under a JEM-1200EX, 80 kv electron microscope (JEOL, Japan).

Histochemistry

Freshly excised rat stomach tissues were fixed with paraformaldehyde fixative (formaldehyde: potassium = 1:4) for 36 h at 4℃. The tissues were then fixed in 3% potassium bichromate fixative for 12 h at room temperature, and embedded in paraffin. Histochemical staining for ANP-synthesizing cells were performed by the chromaffin staining method. After washing twice with distilled water for 20 min, the tissues were stained at room temperature for 20 min with 10 g/L toluidine solution, and after washing twice with distilled water for 10 min, the sections were stained at room temperature for 15 min with 1% saffron solution. Chromaffin staining was made for enterochromaffin cells. The positive cells were expressed as brown granules, which were mainly located in cell cytoplasm under microscopy. At least 5 high-power (× 400 field) fields were chosen randomly for cell observation. The area density of the distribution of positive cells was calculated under light microscopy (Olympus BH-2, Japan) and with CMIAS image analysis system (Beihang, China).

Tannic acid-ferric chloride method

The gastric microvessels in density and distribution were investigated in different regions of stomach. After the experimental rats (Wistar rats) were perfused with 20 g/L compound fixative tannic acid solution, the stomachs were taken out and cut into frozen sections, then these sections were immersed in 20 g/L ferric chloride solution at room temperature for 20 min to reveal the microvessels. The blood vessels were revealed distinctively by TA-Fe staining

method, and observed under the light microscope, the density and distribution of the vessels were measured and analyzed by CMIAS image analysis system. Density and distribution of microvessels in rat stomach was determined according to the KONG Xiang-yu *et al* method[13], and was photomicrographed (Olympus PM-10AD, Japan).

Statistical analysis

Data were expressed as mean ± SD. The two-tailed χ^2 test was used to examine the correlation between ANP-synthesizing cells and microvessel density. Statistical significance was estimated by *t* test. Differences were considered significant when $P < 0.05$. All the calculations were performed using SPSS11.0.

RESULTS

Expression of ANP-synthesizing cells in rat stomach

Immunohistochemical positive expressions of ANP-synthesizing cells were exhibited in paraffin sections of atria and stomach. As a positive control, ANP-synthesizing cells showed intense positive expression in atrial myocytes cytoplasma (as red brown granules, Figure 2A). ANP-synthesizing cells also were positively expressed in gastric mucosa and the positive granules localized to cytoplasm in the basal portion of cardiac region glands (Figure 2B and C). In negative controls, complete absence of positive staining for ANP-synthesizing cells was observed when normal rabbit serum was substituted for primary antiserum (Figure 2D). The morphological shape of the individual ANP-synthesizing cells was variable, exhibiting round, pyramidal, flask shapes, etc. The general epithelial morphology of these cells was typically endocrine in appearance, and most immunoreactivety was localized to the basal portion of the stomach of rats. Negative staining for ANP-synthesizing cells was detected in the lamina propria, submucosa, and smooth muscle. Expression of ANP-synthesizing cells was exhibited in different regions, and its density was the largest in gastric cardiac region, and the density order of ANP-synthesizing cells was cardiac region (cardia) > pyloric region (antrum) > fundic region (fundus) in mucosal layer.

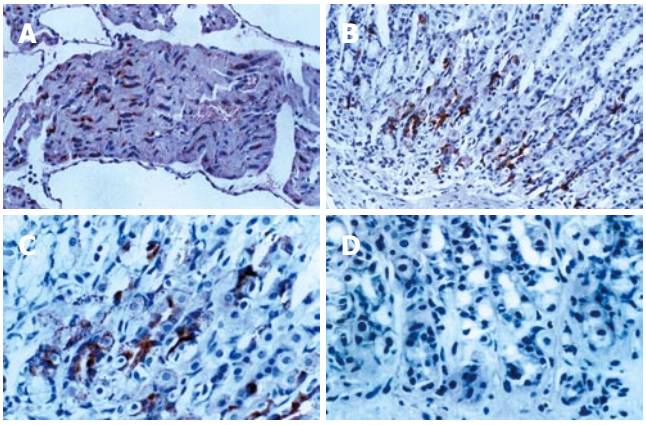


Figure 2 **A**: As a positive control, atrial myocytes show intense positive cytoplasmic staining for ANP (IHC × 200); **B**: Positive staining for ANP is localized to cytoplasm of mucosal cells in cardiac glands (IHC × 200); **C**: Positive staining for ANP is localized to cytoplasm of mucosal cells in cardiac glands (IHC × 400); **D**: As a negative control, complete absence of staining is observed when normal rabbit serum is substituted for ANP (IHC × 400).

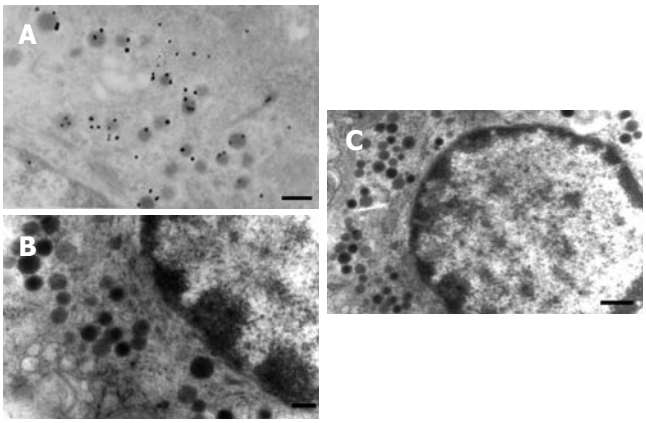


Figure 3 **A**: localization of immunogold labeled in the endocrine granule of the enterochromaffin cell (TEM × 20 000, bar = 200 nm); **B**: Negative control,complete absence of immunogold labeled in the endocrine granule of enterochromaffin cells when normal rabbit serum was substituted for anti-ANP antiserum (TEM × 20 000, Bar = 200 nm); **C**: Normal enterochromaffin cells in gastric mucosa (TEM × 15 000, Bar = 500 nm).

Identification of ANP-synthesizing cells in rat stomach

The gastric mucosa was cut into ultra-thin sections, and situs ultrastructural detection of ANP-synthesizing cells was carried out using a post-embedding immunogold labeling technique under electron microscope. The localization of immunogold labeling was displayed in the endocrine granule of the enterochromaffin (EC) cell of DNES (disperse or diffuse neuroendocrine system, DNES) in gastric gland of rat (Figure 3A). These results indicate that the EC cell synthesizes and secretes ANP in gastric mucosa of rat. In negative control, complete absence of positive staining was observed when normal rabbit serum was substituted for anti-ANP antiserum (Figure 3B and C).

Distribution of ANP-synthesizing cells in rat gastric mucosa

The distribution of ANP-synthesizing cells (enterochromaffin cell, EC cells) in the different regions of gastric mucosa was detected using a histochemical technique in rats. It has been identified that EC cell is only a chromaffin cell in the rat gastric mucosa. Consecutive serial sections were stained for chromaffin and the chromaffin granules (brown granules) were localized in EC cell cytoplasm. Negative staining for chromaffin granules were detected in the lamina propria, submucosa, smooth muscle and in negative control. The distribution of EC cells in gastric mucosa was further examined using chromaffin staining. There were three histologically distinct regions (cardia, fundus and antrum) in the distribution of EC cells in rats. EC cells existed in mucosal layer, and its density was the largest in gastric cardia, and the density order of EC cells (mean ± SD) was cardiac region > pyloric region > fundic region in mucosal layer (Figure 4, $n = 18$).

Relationship between ANP-synthesizing cells and microvessel density

The microvessels were stained successfully with tannic acid-ferric chloride[13,14]. Microvessel of gastric mucous

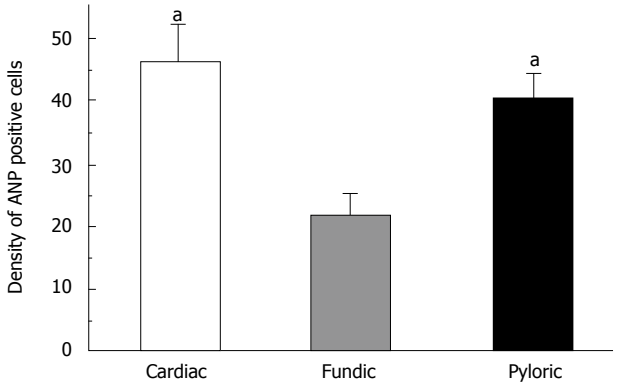
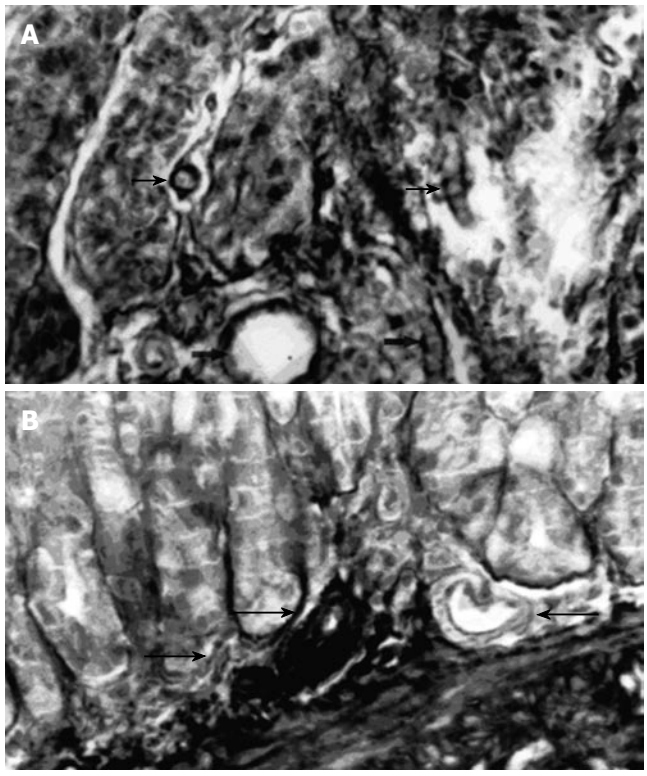


Figure 4 The distribution of ANP synthesizing cells in different regions of stomach in rats. Mean ± SD. $^{a}P < 0.05$ *vs* fundic region.



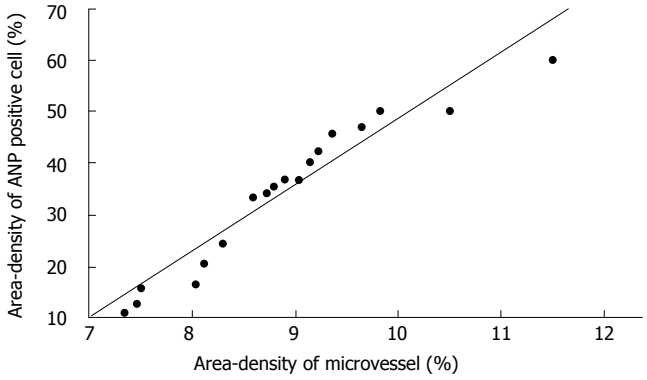


Figure 5 **A**: Microvessels of gastric mucous were in wriggled way and cut into different cross-sections, it could be clearly observed in distinct three-dimensions (arrow) (TA-Fe × 400); **B**: Some branch arteries from the large vessels run into the basal glands of the gastric mucosa (arrow) (TA-Fe × 400); **C**: There is positive significant relationship between the positive rate (%) of ANP-synthesizing cells and microvessel density (%) in cardiac region mucosa of rats ($r = 0.53$, $P < 0.05$, $n = 18$).

were in wriggled way and cut into different cross-sections, it could be clearly observed in distinct three-dimensions (Figure 5A). Some microvessels could be found scattering among antral mucosa, some branch arteries from the large vessels ran into the basal glands of the gastric mucosa (Figure 5B). The density of microvessels varies from position to position, the more basal glands were found, the more microvessel were distributed in rat gastric mucous. In order to study the relationship between the positive rate of ANP-synthesizing cells and microvessel density in rat gastric mucosa, the data were analyzed by statistical analysis system of SPSS11.0. There was a positive significant relationship between the positive rate of ANP-synthesizing cells and microvessel density in antral mucosa of rats ($r = 0.53$, $P < 0.05$, Figure 5C).

DISCUSSION

In the present study, the ANP-expressing myoendocrine cells are most concentrated in the right atrium, to a lesser extent in the left atrium, and almost absent in the left ventricle[15,16].We have demonstrated that morphological distribution and ultrastructural localization of ANP-synthesizing cells in rat stomach. ANP may have tissue-specific functions within the stomach. Gower *et al*[17-19] and Vuolteenaho *et al*[20-24]earlier found that the rat antrum contains ANP-synthesizing cells coupled with our present results that similar immunostaining patterns are produced when antibodies are directed to antral mucosal cells, however we have also found positive cells in cardiac and fundus. EC cells are an abundant type of enteroendocrine cells that contain serotonin and occur throughout the gastrointestinal tract[25-28]. On the basis of differences in the ultrastructural appearance of the secretary granules, it has been suggested that EC cells are comprised of several subpopulations and store different peptides. Three types of EC cells for EC_1, EC_2, and EC_n have been identified. EC cells have two types of open-type enterochromaffin cell and close-type enterochromaffin cell. Open-type enterochromaffin cells have a large basolateral compartment in contact with the basal lamina and a narrow apical process that allows access to the lumen. Results from our present immunohistochemical studies demonstrate that at least some of the ANP-synthesizing cells in the gastric mucosa are exposed to both basal lamina and lumen, and under immunoelectron microscopy they were identified as EC cells that synthesized ANP.

Guo HS *et al*[29] using a radioautograph technique, detected the distribution of NPR (NPR-A) in different regions of rat stomach. NPR-A existed in both the mucosal layer and muscle layer, and the distribution order of NPR-A in density being antrum > body > fundus in muscle layer. Rambotti *et al*[30] found that NPR-A extensively distributed in many tissues, for example, the bladder of the toad, bullfrog brain, in fetal ovine pulmonary vascular and in the porcine coronary. The present study confirmed that NPR-A also existed in the stomach of rat and the density was largest in gastric antrum in rats, demonstrating the presence of NPR-A transcripts in the extracts of gastric fundus and natriuretic peptide-induced cGMP production

localized to the parietal, mucus secreting cells in the fundus, and pyloric glands, as well as gastric smooth muscle cells[29]. Because ANP is known to stimulate gastric acid secretion and relaxes gastric smooth muscle, the effect of atrial natriuretic factor (ANF) on exocrine pancreatic secretion and the possible receptors and pathways involved were studied *in vivo*[29,31]. These findings suggest that these effects of ANP may be direct. Rambotti *et al*[31] demonstrated the presence of ANP-induced guanylate cyclase activity on both apical and basolateral surfaces of mucosal cells within the pyloric glands of rat stomach, ANP released locally into the gastric lumen could target luminally directed receptors, suggesting that ANP may help control a "negative feedback" system within the stomach of increasing acid secretion and simultaneously enchance mucus production to protect the lining of the stomach from the effects of acid. This would provide for a regulatory mechanism to ensure that the acid produced after a meal does not injure the mucosal surcosal surface of the stomach. Previous studies also indicated that vagus nerve regulated many functions of NP, for example, ANP promoted gastric acid secretion[32], ANP and endothelin-1 (ET-1) might prevent renal dysfunction during the progression of congestive heart failure(CHF) through the cGMP pathway in dogs[33,34], ANP can reduce the pre-load and after-load in normal and failing hearts[35-37]. ANP each inhibited the growth of the human pancreatic adenocarcinomas *in vivo* and three of the four peptide hormonesed the volume of the tumors (up to 49%)[10,38,39]. The intestinal tract is a target organ for ANP, characterized by various biologic activities, immunoreactivity, as well as specific binding sites for ANP[40,41].

In conclusion, our result demonstrated that ANP-synthesizing cells exist in gastric mucosa of rats, and its density was largest in gastric cardiac region,and the distribution order of ANP-synthesizing cells in density was cardiac region > antrum > fundic region in mucosa layer. This result shows that it is the EC cells in the gastric mucosa that synthesize ANP. This basolateral plasma membrane is juxtaposed to microvessel *via* ANP which could enter the circulation from the stomach. The density of microvessel varies from position to position at the EC cell concentrating area, and the relationship between distribution of ANP-synthesizing cells and microvessel density was investigated in the stomach of rats.

REFERENCES

1 **McGrath MF**, de Bold ML, de Bold AJ. The endocrine function of the heart. *Trends Endocrinol Metab* 2005; **16**: 469-477
2 **Bensimon M**, Chang AI, de Bold ML, Ponce A, Carreras D, De Bold AJ. Participation of G proteins in natriuretic peptide hormone secretion from heart atria. *Endocrinology* 2004; **145**: 5313-5321
3 **Lai P**, Nazian SJ, Gower WR Jr, Landon CS, Dietz JR. Increased bioactivity of rat atrial extracts: relation to aging and blood pressure regulation. *J Gerontol A Biol Sci Med Sci* 2000; **55**: B390-B395
4 **Lai FJ**, Hsieh MC, Hsin SC, Lin SR, Guh JY, Chen HC, Shin SJ. The cellular localization of increased atrial natriuretic peptide mRNA and immunoreactivity in diabetic rat kidneys. *J Histochem Cytochem* 2002; **50**: 1501-1508
5 **Cayli S**, Ustünel I, Celik-Ozenci C, Korgun ET, Demir R. Distribution patterns of PCNA and ANP in perinatal stages of the developing rat heart. *Acta Histochem* 2002; **104**: 271-277
6 **Schulte I**, Bektas H, Klempnauer J, Borlak J. Vitamin E in heart transplantation: effects on cardiac gene expression. *Transplantation* 2006; **81**: 736-745
7 **Zhao L**, Mason NA, Strange JW, Walker H, Wilkins MR. Beneficial effects of phosphodiesterase 5 inhibition in pulmonary hypertension are influenced by natriuretic Peptide activity. *Circulation* 2003; **107**: 234-237
8 **Madhani M**, Scotland RS, MacAllister RJ, Hobbs AJ Vascular natriuretic peptide receptor-linked particulate guanylate cyclases are modulated by nitric oxide-cyclic GMP signalling. *Br J Pharmacol* 2003; **139**: 1289-1296
9 **Peng N**, Chambless BD, Oparil S, Wyss JM. Alpha2A-adrenergic receptors mediate sympathoinhibitory responses to atrial natriuretic peptide in the mouse anterior hypothalamic nucleus. *Hypertension* 2003; **41**: 571-575
10 **Saba SR**, Vesely DL. Cardiac natriuretic peptides: hormones with anticancer effects that localize to nucleus, cytoplasm, endothelium, and fibroblasts of human cancers. *Histol Histopathol* 2006; **21**: 775-783
11 **Gower WR Jr**, Salhab KF, Foulis WL, Pillai N, Bundy JR, Vesely DL, Fabri PJ, Dietz JR. Regulation of atrial natriuretic peptide gene expression in gastric antrum by fasting. *Am J Physiol Regul Integr Comp Physiol* 2000; **278**: R770-R780
12 **Gower WR Jr**, San Miguel GI, Carter GM, Hassan I, Farese RV, Vesely DL. Atrial natriuretic hormone prohormone gene expression in cardiac and extra-cardiac tissues of diabetic Goto-Kakizaki rats. *Mol Cell Biochem* 2003; **252**: 263-271
13 **Kong XY**, Zhao SM, Yang FG, Ma Q, Zhao LX, Liu S, Du SJ, Du JK. Quantitative investigations of microvessels by TA-Fe method in different regions of crerebrum. *Progr Anat Sci* 2004; **10**: 100-102
14 **Ma Q**, Zhao SM, Kong XY, Yan R,Liu S.Observation of pulmonary microvasculature and cells with Tannic Acid-Ferric chloride (TA-Fe) staining under the Light Microscope. *Jiepou Kexue Jinzhan* 2003; **9**: 230-232
15 **Osman AH**, Yuge S, Hyodo S, Sato S, Maeda S, Marie H, Caceci T, Birukawa N, Urano A, Naruse K, Naruse M, Takei Y. Molecular identification and immunohistochemical localization of atrial natriuretic peptide in the heart of the dromedary camel (Camelus dromedarius). *Comp Biochem Physiol A Mol Integr Physiol* 2004; **139**: 417-424
16 **Peters CG**, Miller DF, Giovannucci DR. Identification, localization and interaction of SNARE proteins in atrial cardiac myocytes. *J Mol Cell Cardiol* 2006; **40**: 361-374
17 **Gower WR Jr**, Dietz JR, Vesely DL, Finley CL, Skolnick KA, Fabri PJ, Cooper DR, Chalfant CE. Atrial natriuretic peptide gene expression in the rat gastrointestinal tract. *Biochem Biophys Res Commun* 1994; **202**: 562-570
18 **Poulos JE**, Gower WR, Fontanet HL, Kalmus GW, Vesely DL. Cirrhosis with ascites: increased atrial natriuretic peptide messenger RNA expression in rat ventricle. *Gastroenterology* 1995; **108**: 1496-1503
19 **Poulos JE**, Gower WR Jr, Sullebarger JT, Fontanet HL, Vesely DL. Congestive heart failure: increased cardiac and extracardiac atrial natriuretic peptide gene expression. *Cardiovasc Res* 1996; **32**: 909-919
20 **Vuolteenaho O**, Arjamaa O, Vakkuri O, Maksniemi T, Nikkilä L, Kangas J, Puurunen J, Ruskoaho H, Leppäluoto J. Atrial natriuretic peptide (ANP) in rat gastrointestinal tract. *FEBS Lett* 1988; **233**: 79-82
21 **Novaira HJ**, Ornellas DS, Ortiga-Carvalho TM, Zhang XM, Souza-Menezes J, Guggino SE, Guggino WB, Morales MM. Atrial natriuretic peptide modulates cystic fibrosis transmembrane conductance regulator chloride channel expression in rat proximal colon and human intestinal epithelial cells. *J Endocrinol* 2006; **189**: 155-165
22 **Gower WR Jr**, Premaratne S, McCuen RW, Arimura A, McAfee Q, Schubert ML. Gastric atrial natriuretic peptide regulates endocrine secretion in antrum and fundus of human and rat stomach. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G638-G645
23 **Gower WR Jr**, McCuen RW, Arimura A, Coy DA, Dietz JR,

Landon CS, Schubert ML. Reciprocal paracrine pathways link atrial natriuretic peptide and somatostatin secretion in the antrum of the stomach. *Regul Pept* 2003; **110**: 101-106

24 **Gower WR Jr**, Dietz JR, McCuen RW, Fabri PJ, Lerner EA, Schubert ML. Regulation of atrial natriuretic peptide secretion by cholinergic and PACAP neurons of the gastric antrum. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G68-G74

25 **Sundler F**, Böttcher G, Ekblad E, Håkanson R. The neuroendocrine system of the gut. *Acta Oncol* 1989; **28**: 303-314

26 **Zaiachkivs'ka OS**, Hzhehots'kyĭ MR, Kovalyshyn VI. [Oral and gastric diffuse neuroendocrine system: discussion questions of structure and function]. *Fiziol Zh* 2005; **51**: 79-90

27 **Dinan TG**, Quigley EM, Ahmed SM, Scully P, O'Brien S, O'Mahony L, O'Mahony S, Shanahan F, Keeling PW. Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* 2006; **130**: 304-311

28 **Smith SM**, Vaughan JM, Donaldson CJ, Fernandez RE, Li C, Chen A, Vale WW. Cocaine- and amphetamine-regulated transcript is localized in pituitary lactotropes and is regulated during lactation. *Endocrinology* 2006; **147**: 1213-1223

29 **Guo HS**, Cui X, Cui YG, Kim SZ, Cho KW, Li ZL, Xu WX. Inhibitory effect of C-type natriuretic peptide on spontaneous contraction in gastric antral circular smooth muscle of rat. *Acta Pharmacol Sin* 2003; **24**: 1021-1026

30 **Rambotti MG**, Giambanco I, Spreca A. Detection of guanylate cyclases A and B stimulated by natriuretic peptides in gastrointestinal tract of rat. *Histochem J* 1997; **29**: 117-126

31 **Sabbatini ME**, Villagra A, Davio CA, Vatta MS, Fernandez BE, Bianciotti LG. Atrial natriuretic factor stimulates exocrine pancreatic secretion in the rat through NPR-C receptors. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G929-G937

32 **Guo HS**, Jin Z, Jin ZY, Li ZH, Cui YF, Wang ZY, Xu WX. Comparative study in the effect of C-type natriuretic peptide on gastric motility in various animals. *World J Gastroenterol* 2003; **9**: 547-552

33 **Yamamoto T**, Wada A, Ohnishi M, Tsutamoto T, Fujii M, Matsumoto T, Takayama T, Wang X, Kurokawa K, Kinoshita M. Chronic administration of phosphodiesterase type 5 inhibitor suppresses renal production of endothelin-1 in dogs with congestive heart failure. *Clin Sci (Lond)* 2002; **103** Suppl 48: 258S-262S

34 **Vesely DL**. Which of the cardiac natriuretic peptides is most effective for the treatment of congestive heart failure, renal failure and cancer? *Clin Exp Pharmacol Physiol* 2006; **33**: 169-176

35 **Woods RL**. Cardioprotective functions of atrial natriuretic peptide and B-type natriuretic peptide: a brief review. *Clin Exp Pharmacol Physiol* 2004; **31**: 791-794

36 **Nishikimi T**, Maeda N, Matsuoka H. The role of natriuretic peptides in cardioprotection. *Cardiovasc Res* 2006; **69**: 318-328

37 **Yamada T**, Murakami Y, Okada T, Okamoto M, Shimizu T, Toyama J, Yoshida Y, Tsuboi N, Ito T, Muto M, Kondo T, Inden Y, Hirai M, Murohara T. Plasma atrial natriuretic Peptide and brain natriuretic Peptide levels after radiofrequency catheter ablation of atrial fibrillation. *Am J Cardiol* 2006; **97**: 1741-1744

38 **Vesely DL**, Clark LC, Garces AH, McAfee QW, Soto J, Gower WR Jr. Novel therapeutic approach for cancer using four cardiovascular hormones. *Eur J Clin Invest* 2004; **34**: 674-682

39 **Vesely DL**. Atrial natriuretic peptides: anticancer agents. *J Investig Med* 2005; **53**: 360-365

40 **Hervieu G**, Volant K, Grishina O, Descroix-Vagne M, Nahon JL. Similarities in cellular expression and functions of melanin-concentrating hormone and atrial natriuretic factor in the rat digestive tract. *Endocrinology* 1996; **137**: 561-571

41 **González Bosc LV**, Majowicz MP, Vidal NA. Effects of atrial natriuretic peptide in the gut. *Peptides* 2000; **21**: 875-887

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CLINICAL RESEARCH

Mutifactorial analysis of risk factors for reduced bone mineral density in patients with Crohn's disease

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Abstract

AIM: To determine the prevalence of osteoporosis in a cohort of patients with Crohn's disease (CD) and to identify the relative significance of risk factors for osteoporosis.

METHODS: Two hundred and fifty-eight unselected patients (92 M, 166 F) with CD were studied. Bone mineral density (BMD) was measured at the lumbar spine and hip by dual X-ray absorptiometry. Bone formation was assessed by measuring bone specific alkaline phosphatase (BSAP) and bone resorption by measuring urinary excretion of deoxypyridinoline (DPD) and N-telopeptide (NTX).

RESULTS: Between 11.6%-13.6% patients were osteoporotic (T score < -2.5) at the lumbar spine and/or hip. NTX levels were significantly higher in the patients with osteoporosis ($P < 0.05$) but BSAP and DPD levels were not significantly different. Independent risk factors for osteoporosis at either the lumbar spine or hip were a low body mass index ($P < 0.001$), increasing corticosteroid use ($P < 0.005$), and male sex ($P < 0.01$). These factors combined accounted for 23% and 37% of the reduction in BMD at the lumbar spine and hip respectively.

CONCLUSION: Our results confirm that osteoporosis is common in patients with CD and suggest that increased bone resorption is the mechanism responsible for the bone loss. However, less than half of the reduction in BMD can be attributed to risk factors such as corticosteroid use and low BMI and therefore remains unexplained.

Key words: Crohn's disease; Osteoporosis; Osteopenia; Bone mineral density

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INTRODUCTION

Osteoporosis is now recognised as a common complication of inflammatory bowel disease (IBD) and in particular Crohn's disease (CD). Estimates of prevalence vary but those studies employing the World Health Organisation[1] diagnostic criteria (a bone density 2.5 or more standard deviation units below the mean value for young adults) report rates of 13%-42%[2-4]. The pathogenesis of osteoporosis in patients with CD is likely to be multifactorial. Most, but not all studies, have found an association with current or cumulative corticosteroid use[5-11] but other factors such as disease duration[3,4,12] low body weight or body mass index[4,10,13] calcium and vitamin D deficiency[12,14] small bowel involvement or resection[10] smoking[15] gender and increasing age[13] have been implicated. At the molecular level *in vitro* studies have demonstrated that serum from children with CD can inhibit bone formation suggesting that pro-inflammatory cytokines such as IL-6 may be involved[16].

The mechanism of bone loss is poorly understood. Normal bone in healthy adults is in a state of equilibrium, the rate of osteoblastic bone formation equaling the rate of bone resorption by osteoclasts. Biochemical markers of bone turnover such as deoxypyridinoline (DPD) and cross-linked N-telopeptides of type 1 collagen (Ntx), both markers of bone resorption, and osteocalcin and bone specific alkaline phosphatase (BSAP), markers of bone formation, are now available and have been used in several studies examining the possible mechanisms of bone loss in patients with IBD. Consistent findings are raised levels

of either DPD or Ntx[2,4,12,17,18] suggesting increase in bone resorption, although there is also evidence that bone formation is reduced[7].

The aim of this cross sectional study was to determine the prevalence of osteoporosis in an unselected group of patients with CD and to identify the relative importance of possible risk factors and the mechanism of bone loss.

MATERIALS AND METHODS

Patients were recruited from the IBD register at the Freeman Hospital and additionally from the Royal Victoria Infirmary, also in Newcastle-upon-Tyne, and the Queen Elizabeth Hospital, Gateshead. Patients were under the care of either surgical or medical gastroenterologists and all fulfilled at least two of four diagnostic criteria (histological, radiographic, endoscopic and surgical)[19]. Patients were contacted by letter inviting them to participate in the study and were then seen at the Freeman Hospital by either SB or NT. Those who did not attend were sent one further letter reminding them to do so. We included patients aged between 25 and 70 years only and excluded women who were pregnant or planning a pregnancy because of the potential risk from exposure to ionizing radiation during bone densitometry.

A questionnaire was completed with the patient detailing age, tobacco and alcohol consumption, fracture history, and in women, reproductive and menstrual history. Details of duration and site of disease, corticosteroid use (expressed as number of months on corticosteroids), relevant surgical history and drug history were obtained at interview and by careful scrutiny of the medical notes. Height and weight was measured immediately prior to bone densitometry and these figures were used to calculate body mass index [weight/height^2, (kg/m^2)]. The study was approved by the Newcastle-upon-Tyne Joint Ethics Committee.

Bone mineral density measurements

Bone mineral density (BMD) was measured at the lumbar spine (L1-L4) and left hip (total hip) by dual X-ray absorptiometry (Hologic Inc. QDR 2000, Waltham, MA.). The coefficient of variation (CV) is 0.7% at the lumbar spine and 1.0% at the hip[20]. BMD results are expressed as an areal density in g/cm^2, but have been compared with the manufacturer's mean value for young adults and for normal people of the same age and sex to give T scores and Z scores respectively. The T score is the number of standard deviation units above or below the mean value for young adults of the same sex, whilst the Z score is the number of standard deviation units above or below the age related mean value. The WHO has defined osteopenia as a T score of < -1 but > -2.5, whilst osteoporosis is defined as a T score of -2.5 or lower. These diagnostic criteria are not necessarily thresholds for intervention, particularly for patients on oral corticosteroids, where a higher T score of -1.5 would be more appropriate[21].

Biochemical measurements

Serum and urine samples were taken between 2 pm and 4 pm. The serum was immediately centrifuged and all samples were stored at -30℃. Serum calcium and phosphate levels were measured using standard methods on an Olympus 600 automated system, (interassay CVs for calcium and phosphate are < 1% and < 2% respectively).

Bone resorption was assessed by measurement of the urinary excretion of free DPD and Ntx with values expressed as a fraction of urinary creatinine excretion. Free DPD was measured by competitive immunoassay (Chiron Diagnostics Corporation, MA) (interassay CV < 8%). The reference range (manufacturer's data), is 3.0-7.4 nmol DPD/mmol creatinine for females aged 25-44 years and 2.3-5.4 nmol DPD/mmol creatinine for males aged 25-55 years. Ntx was measured by enzyme-linked immunoabsorbant assay ("Osteomark", Ostex International, Seattle, WA) with results expressed as bone collagen eqivalents (BCE). The reference ranges for men and women (manufacturer's data) are 3-51 and 5-65 nm/mmol creatinine respectively (interassay CVs < 9%). Bone formation was assessed by measurement of serum BSAP using enzyme-linked immunoabsorbant assay (Metra BioSystems, Southampton, UK) (interassay CV < 6%).

Patients with a T score of -1.5 or less at either the lumbar spine were investigated further to look for other secondary causes of osteoporosis, such as vitamin D deficiency with secondary hyperparathyroidism, thyroid disease and, in men, hypogonadism.

Intact parathyroid hormone (PTH) levels, reference range 12-72 ng/L, were measured by immunometric assay (Immulite Intact PTH, Diagnostics Products Corporation, Los Angeles, CA.) (interassay CV < 5%). Vitamin D levels (25 OH Cholecalciferol and 25 OH Ergocalciferol), normal range 10-50 nmol/L, were assayed by high performance liquid chromatography performed at the Department of Clinical Biochemistry, Royal Victoria Infirmary, Newcastle-upon-Tyne (interassay CV < 12%). Thyroid stimulating hormone (TSH), reference range 0.3-4.1 Mu/L, was measured with a two site immunoassay using direct chemiluminometric detection and monoclonal/polyclonal antibodies (interassay CV < 4.0%). Testosterone, reference range 9-20 nmol/L, was measured with a competitive immunoassay using direct chemiluminometric detection and a polyclonal antibody (interassay CV < 5.2%) and Sex Hormone Binding Globulin (SHBG) was measured with a non-competitive immunoradiometric assay (interassay CV < 5%). The free androgen index (FAI), reference range > 3 nmol/nmol, was calculated after dividing the total serum testosterone by the SHBG level.

Statistical analysis

The results are expressed as means ± SD. Comparison between group means was anlaysed using Student's unpaired *t*-test or, where data was not normally distributed, the Mann-Whitney *U*-test. Multiple regression analysis was performed to determine independent risk factors for osteoporosis and the adjusted χ^2 value was calculated to determine the proportion of the variability in BMD attributable to these factors. The chi-squared test was used to compare incidences. A *P* value of < 0.05 was taken to be statistically significant. Analyses were made with

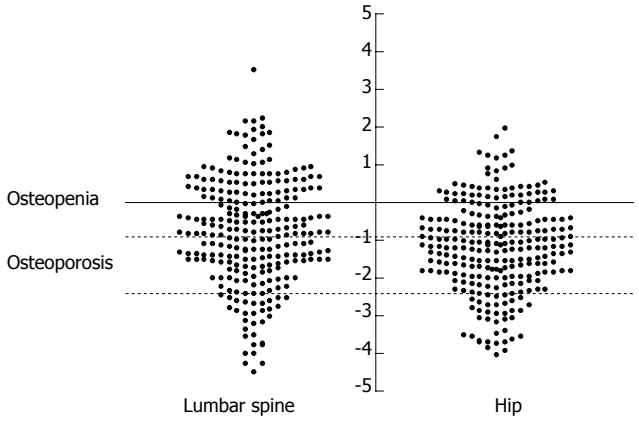

Figure 1 Scatterplot of T score values at the lumbar spine and hip. Dotted lines represent thresholds for diagnosis of osteopenia and osteoporosis.

GraphPad Prism (GraphPad Software Inc) and Minitab (Version 12.1, Minitab Inc).

RESULTS

Two hundred and sixty five patients responded to the correspondence inviting them to participate in the study, 7 of whom subsequently declined a DXA scan and were excluded from the study. The remaining 258 patients who completed the study comprised 92 men and 166 women. One patient was of Asian descent, the remainder were white. Table 1 summarizes the clinical characteristics of the group.

Fractures

Twenty-eight patients (10.9%) reported a low trauma fracture in adulthood, 12 of whom had osteoporosis. The fractures comprised wrist[9], forearm[5] and vertebral[3] fractures, and 2 each of metatarsal, rib and ankle fractures, 1 patella fracture and 1 femoral fracture. Two patients had more than 1 fracture and in 5 cases the fracture site was not documented.

Site of disease

One hundred and ninety two (74.4%) had small bowel disease, of which 70 (27.1%) had small bowel involvement alone and 88 (34.1%) had small bowel involvement with colonic disease. Forty-nine (18.9%) had isolated colonic disease. At least one partial small bowel resection had been performed in 135 (52.3%) of the study group, whilst 92 (35.7%) had undergone partial or total resection of their large bowel.

Drug treatment

Seventy-seven patients (29.8%) were currently taking oral corticosteroids and 152 (58.9%) had previously taken corticosteroids to treat complications of their Crohn's disease. Forty-five patients (17.4%) were currently taking bone active treatment (calcium supplementation with or without vitamin D, bisphosphonates or hormone replacement therapy).

Table 1 Patient characteristics (means ± SD)

n	258 (166 F, 92 M)
Age (yr)	44.5 (± 11.5)
Postmenopausal women	67 (25.9%)
Fractures	28 (10.8%)
BMI (kg/m^2)	24.7 (± 4.6)
Disease duration (yr)	14.3 (± 9.5)
Small bowel involvement	192 (74.4%)
Small bowel resection	135 (52.3%)
Large bowel involvement	173 (67.1%)
Current CS use	77(29.8%)
Previous CS use	152 (58.9%)
Duration CS use (mo)	51.0 (± 68.4)
Current smokers	83 (32.2%)

CS: Corticosteroids.

Table 2 Bone mineral density measurements (means ± SD)

Site	BMD (g/cm^2)	Z score	T score	Osteopenia	Osteoporosis
Lumbar spine	0.975 ± 0.15	-0.308 ± 1.39	-0.795 ± 1.39	77 (29.8%)	30 (11.6%)
Total hip	0.860 ± 0.15	-0.675 ± 1.19	-1.181 ± 1.21	67 (25.9%)	35 (13.6%)

Osteopoenia defined as a T score of < -1 but > –2.5; Osteoporosis defined as a T score of < -2.5.

Tobacco and alcohol consumption

Eighty-three patients (32.2%) were current cigarette smokers and 78 (30.2%) previous smokers. The mean alcohol intake was 8.8 units/week among the 160 patients who drank alcohol.

Bone mineral density measurements

Measurements at the lumbar spine were available in 257 patients, one was excluded because of the presence of surgical metal rods. Measurements at the hip were available in all 258 patients. Details of the mean BMD measurements, mean Z and T scores and numbers with osteopenia or osteoporosis are presented in Table 2 and Figure 1. Employing the WHO definitions for osteoporosis 18 (6.9%) were osteoporotic at both the lumbar spine and hip. Considering each site separately, 30 (11.6%) patients were osteoporotic at the lumbar spine (16 men, 14 women) and 35 (13.6%) were osteoporotic at the hip (15 men, 20 women) whilst a further 77 (29.8%) and 67 (25.9%) fulfilled the criteria for osteopenia at the lumbar spine and hip respectively.

Risk factors

Table 3 shows the characteristics of those patients with and without osteoporosis. At the lumbar spine patients with osteoporosis had a significantly lower BMI and had taken corticosteroids for longer, there was a trend towards longer disease duration although this did not achieve statistical significance. Patients with osteoporosis at the hip were also likely to have a lower BMI, a longer disease duration and have taken corticosteroids for longer. Gender, small bowel resection and current tobacco use were not

Table 3 Comparison of those patients with and without osteoporosis and the presence of risk factors (mean ± SD)

Risk factor	Osteoporosis at lumbar spine and/or hip (*n* = 47)	Osteopenia/ normal bone mineral density (*n* = 211)	*P*
BMI (kg/m^2)	22.3 ± 0.6	25.2 ± 0.3	< 0.0001
Corticosterid use (mo)	92.3 ± 16.7	34.9 ± 3.2	< 0.0001
Disease duration (yr)	18.9 ± 1.5	13.2 ± 0.6	< 0.005
Age (yr)	46.8 ± 11.4	44.1 ± 11.5	NS
Small bowel resection (Y/N)	10/37	105/106	NS
Current smokers (Y/N)	11/36	72/139	NS
Gender (M/F)	22/25	69/142	NS

Analysis was made using Student's unpaired *t*-test or the chi-squared test where appropriate; NS: not significant.

significant factors at either site. One hundred and fifteen patients (44.6%) had T scores < -1.5 at the lumbar spine and/or hip respectively. The T score within individual patients was significantly lower at the hip compared with the lumbar spine (-0.80 ± 1.4 *vs* -1.19 ± 1.2, $P < 0.0001$).

Postmenopausal women had significantly lower T scores at the lumbar spine and hip compared to the rest of the study group (-1.30 ± 0.16 *vs* -0.63 ± 0.10 and -1.5 ± 0.14 *vs* -1.09 ± 0.09 respectively, $P < 0.0001$). This group accounted for 11/29 patients with osteoporosis at the spine and 12/34 at the hip.

Multivariate analysis was used to determine independent predictors for the presence of osteoporosis. Variables included in the analysis were age, sex, disease duration, BMI, length of corticosteroid use and small bowel resection. Of these, decreasing BMI ($P < 0.001$), length of corticosteroid use ($P = 0.003$) and male sex ($P = 0.009$) were the significant predictors at the lumbar spine whilst at the hip decreasing BMI ($P < 0.001$), number of months treatment with corticosteroids ($P < 0.001$), increasing age ($P = 0.003$) and male sex ($P = 0.005$) were significant. The adjusted r^2 value was calculated using sex, age, sex-age interaction, corticosteroid use, BMI, disease duration and small bowel resection (Yes/No) as variables. These factors accounted for 22.9% and 37.2% in the variability in BMD at the lumbar spine and hip respectively.

Biochemical measurements

The mean levels of total calcium and phosphate were normal (Table 4). There was no difference in the means between those with and those without osteoporosis. Patients with a T score < -1.5 at either site ($n = 115$) were investigated further and in these patients mean levels of TSH and 25 OHD were normal (Table 5). Five patients had abnormal thyroid function tests. Two patients had low TSH levels, one of these patients was on thyroid replacement therapy. Of the three with above normal TSH levels, none had abnormal tri-iodothyrine or thyroxine levels. Two patients had biochemically low 25 OHD levels (defined as < 10 mol/L) but neither was associated with a secondary hyperparathyroidism or with small bowel resection. Mean PTH levels were normal. PTH levels were raised (defined as twice the upper limit of normal) in 4 patients (range 153-613 ng/L) including one patient with severe nutritional deficiency (BMI < 19, anaemia, hypoalbuminaemia) and severe osteoporosis who nevertheless had normal 25 OHD levels because of treatment with vitamin D metabolites prior to inclusion into the study. Three men (6.5%) were hypogonadal (FAI < 3 nmol/nmol).

Table 4 Biochemistry and markers of bone formation and resorption in patients with osteoporosis at either the lumbar spine and/or hip compared with those patients with osteopenia or normal bone density (mean ± SD)

Biochemistry (normal range)	Osteoporosis at lumbar spine and/or hip (*n* = 47)	Osteopenia/ normal bone mineral density (*n* = 211)	*P*
Total calcium (2.12-2.55 mmol/L)	2.30 ± 0.03	2.31 ± 0.01	NS
Phosphate (0.65-1.3 mmol/L)	1.11 ± 0.03	1.11 ± 0.03	NS
BSAP (11.6-30.6 μg/L)	19.7 ± 1.4	18.6 ± 0.6	NS
DPD/creat (2.3-7.4 nmol/mmol)	5.8 ± 1.0	5.6 ± 0.4	NS
Ntx/creat (3.0-65.0 BCE)	63.7 ± 9.5	40.6 ± 3.6	$P < 0.05$

BSAP: Bone specific alkaline phosphatase; DPD: Deoxypyridinoline; Ntx: N-telopeptides of type 1 collagen; BCE: Bone collagen eqivalents; NS: not significant.

Table 5 Results of further bone biochemistry carried out in patients with a T score of -1.5 or less at either the lumbar spine and/or hip (*n* = 115) (mean ± SD)

Biochemistry (normal range)	T score < -1.5 at lumbar spine and/or hip (*n* = 115)
TSH (0.3-4.1 Mu/L)	1.7 ± 1.1
25 OHD (10-75 nmol/L)	45.1 ± 22.3
PTH (10-72 ng/L)	46.9 ± 62.7
[1]Testosterone (9-20 nmol/L)	13.5 ± 6.7
[2]FAI (> 0.3 nmol/nmol)	0.4 ± 0.1

[1]Testosterone and SHBG only measured in men ($n = 46$). [2]Free Androgen Index (Testosterone/SHBG).

Markers of bone formation and bone resorption

Mean levels of BSAP and DPD were normal, there was no significant difference between those with and without osteoporosis (Table 4). Ntx levels, although also within the normal range, were significantly higher in patients with osteoporosis.

DISCUSSION

We have shown that between 11%-13% of a large group of unselected patients with CD are osteoporotic. A low BMI, increasing exposure to corticosteroids and male sex appear to be the main predictors of osteoporosis which, however, only explain between 23%-37% of the variability in BMD described. Biochemical abnormalities such as 25 OHD deficiency and secondary hyperparathyroidism,

thyroid disease and male hypogonadism are unusual but our results suggest that increased bone resorption, assessed by measuring urinary excretion of DPD and Ntx, may be the mechanism responsible for the accelerated bone loss.

As far as we are aware this is the largest and most complete study to date of the prevalence of osteoporosis in patients with CD. Our group of patients are unselected and we consider a reasonable representation of the larger population with CD in terms of age and sex composition, duration and site of disease and disease management. Between 11%-13% of our patients are osteoporotic. This is perhaps lower than we expected given the unselected nature of our patient group, although broadly in keeping with other studies using the same diagnostic criteria. However, there is considerable discrepancy among prevalence rates reported. This may be accounted for by differences in the site and the methods used to measure BMD and in the patient groups studied, some for example including those not only with CD but also ulcerative colitis (UC), where there is evidence that osteoporosis is less common[9,22]. Other groups have studied patients with CD only and have selected further to exclude for example those currently taking corticosteroids or at risk of metabolic bone disease[2].

We found that the BMD at the hip was significantly lower compared with the lumbar spine. This has previously been reported in other studies of patients with IBD[2-4,8,23] and is also the pattern of bone loss seen in other chronic inflammatory conditions such as rheumatoid arthritis. It may reflect a degree of degenerative change at the lumbar spine in some of the older patients which would spuriously elevate the density at that site, however we attempted to minimise this effect by excluding patients over the age of 70.

The clinical significance of osteoporosis is the increased susceptibility to fracture and the resulting morbidity and mortality. Eleven percent of our patients reported low trauma fractures, mostly of the upper limb. We did not perform radiography in our patients and, as many vertebral fractures are asymptomatic, the true prevalence is likely to be higher. Relatively few studies have examined fracture incidence and prevalence in adult patients with CD. In retrospective studies, fracture prevalence, calculated from plain radiography of symptomatic areas or self reported fracture, varies between 7% and 27%[7,9,12]. Vestergaard *et al*, in a study of over 800 patients with IBD[24] conducted *via* a postal questionnaire, reported an increased relative risk of low trauma fractures in women with CD of 2.5. The increase in fracture risk was seen mostly in premenopausal women but men with CD or patients of either sex with UC were not at increased risk of fracture. The authors speculated that their findings were due to a number of factors including the increased use of continuous corticosteroids and more severe systemic inflammation in CD compared to UC, and possible hypogonadism in the female patients.

A low BMI, increasing corticosteroid use, male sex and increasing age were independent risk factors in predicting those with osteoporosis although these factors together only accounted for between 20%-40% of the variability in BMD. Low body weight or a low BMI have been reported in other studies of patients with IBD to be significant risk factors for osteoporosis, particularly at the peripheral sites[4-6,9-11]. Within the context of IBD a low BMI may merely reflect severe inflammatory disease and/or malabsorption although there is also good evidence that low body mass *per se* is a significant factor in determining BMD[25,26].

In our study male sex was a significant independent risk factor for osteoporosis at both sites. Most groups have reported no significant differences between the sexes and only one study has looked specifically at men with IBD. Robinson et al studied the hormone profile of 48 men with CD[27] and found biochemical evidence of hypogonadism in only 3 patients, 2 of whom had osteopenia. Three of our male patients (6.5%) with reduced BMD were hypogonadal. This is lower than we might expect given the number of our patients taking corticosteroids which might lead to suppression of gonadotrophin, testosterone and SHBG levels.

The role of corticosteroids in the pathogenesis of osteoporosis in patients with IBD is complex. Whilst some studies have shown a clear relationship between lifetime corticosteroid dose and vertebral fracture rate[5] or low BMD other studies have suggested that BMD is unrelated to corticosteroid use[2,3,6,10]. There have been several prospective studies examining the role of corticosteroids in the rate of bone loss[22,28,29]. The largest of these[28], a longitudinal study of over 100 patients with IBD and bowel resection who were followed up for a mean period of over 5 years found no relationship between bone loss and corticosteroid use. The conflicting results are probably a reflection of the heterogeneity of patient groups studied and the complex relationship between disease severity, systemic inflammation and treatment with corticosteroids.

We found a very low prevalence of biochemical abnormalities in our patients and in particular no evidence of 25 OHD deficiency and secondary hyperparathyroidism or of a correlation with small bowel resection. The relationship between 25 OHD levels and BMD in patients with IBD is not clear cut. Andreassen[14] reporting one of the higher prevalences of 25 OHD deficiency (44%) in a study of 115 patients with CD nevertheless found that this, in combination with PTH levels, only accounted for 4% of the variation in BMD. Croucher[30] described the histomorphometric findings in a study of 19 patients with IBD, all of whom had osteoporosis and 16 of whom had undergone bowel resection. Despite only one patient having a low 25 OHD level, there was evidence of a mild mineralisation defect in the patient group compared with the control group, although none had the classical changes associated with osteomalacia.

Biochemical markers of bone formation and resorption are now widely available and have been used to predict bone loss[31,32] and response to treatment of osteoporosis with bisphosphonates and hormone replacement therapy (HRT) in postmenopausal women[33,34]. Several studies have now used these markers in exploring the mechanisms of bone loss in patients with IBD. Most studies have demonstrated an increase in bone resorption without a corresponding increase in bone formation[2,4,12,17,18]. Our results, which show that patients with osteoporosis

have significantly higher levels of Ntx (but not DPD), would support the results of these studies. It should be noted however that the mean value, even in patients with osteoporosis, was within the normal range and their role in predicting those patients with IBD at risk of losing bone is not clear. Pollak et al has reported that increased levels of Ntx predicted rates of bone loss in a prospective study of 36 patients with IBD over a period of 2 years[35] whilst Schulte[29] found that they did not discriminate between those with accelerated bone loss and those without. Despite this they may be useful in targeting those patients to treat more aggressively with anti-resorptive agents such as HRT and the bisphosphonates.

Management of patients with IBD and osteoporosis remains problematic although recent guidelines for the management and prevention of osteoporosis in IBD have been published in the United Kingdom[36]. The guidelines recommend, amongst other measures, that all patients currently taking corticosteroids and with a T score of < -1.5 should be prescribed a bisphosphonate in addition to vitamin D supplementation. This study highlights the potential clinical implications of this strategy, as approximately 20% of our patients would require bisphosphonates if these guidelines were implemented.

We have also established that although there are several significant risk factors for osteoporosis, these combined explain less than 40% of the variability in BMD. A genetic predisposition to osteoporosis complicating other chronic conditions such as rheumatoid arthritis has been described[37]. This may be of significance in our population and merits further investigation.

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REFERENCES

1 **Kanis JA**. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. *Osteoporos Int* 1994; **4**: 368-381
2 **Bjarnason I**, Macpherson A, Mackintosh C, Buxton-Thomas M, Forgacs I, Moniz C. Reduced bone density in patients with inflammatory bowel disease. *Gut* 1997; **40**: 228-233
3 **Pollak RD**, Karmeli F, Eliakim R, Ackerman Z, Tabb K, Rachmilewitz D. Femoral neck osteopenia in patients with inflammatory bowel disease. *Am J Gastroenterol* 1998; **93**: 1483-1490
4 **Schulte C**, Dignass AU, Mann K, Goebell H. Reduced bone mineral density and unbalanced bone metabolism in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 1998; **4**: 268-275
5 **Compston JE**, Judd D, Crawley EO, Evans WD, Evans C, Church HA, Reid EM, Rhodes J. Osteoporosis in patients with inflammatory bowel disease. *Gut* 1987; **28**: 410-415
6 **Pigot F**, Roux C, Chaussade S, Hardelin D, Pelleter O, Du Puy Montbrun T, Listrat V, Dougados M, Couturier D, Amor B. Low bone mineral density in patients with inflammatory bowel disease. *Dig Dis Sci* 1992; **37**: 1396-1403
7 **Abitbol V**, Roux C, Chaussade S, Guillemant S, Kolta S, Dougados M, Couturier D, Amor B. Metabolic bone assessment in patients with inflammatory bowel disease. *Gastroenterology* 1995; **108**: 417-422
8 **Silvennoinen JA**, Karttunen TJ, Niemelä SE, Manelius JJ, Lehtola JK. A controlled study of bone mineral density in patients with inflammatory bowel disease. *Gut* 1995; **37**: 71-76
9 **Jahnsen J**, Falch JA, Aadland E, Mowinckel P. Bone mineral density is reduced in patients with Crohn's disease but not in patients with ulcerative colitis: a population based study. *Gut* 1997; **40**: 313-319
10 **Robinson RJ**, al-Azzawi F, Iqbal SJ, Kryswcki T, Almond L, Abrams K, Mayberry JF. Osteoporosis and determinants of bone density in patients with Crohn's disease. *Dig Dis Sci* 1998; **43**: 2500-2506
11 **Martin JP**, Tonge KA, Bhonsle U, Jacyna MR, Levi J. Bone mineral density in patients with inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 1999; **11**: 537-541
12 **Bischoff SC**, Herrmann A, Göke M, Manns MP, von zur Mühlen A, Brabant G. Altered bone metabolism in inflammatory bowel disease. *Am J Gastroenterol* 1997; **92**: 1157-1163
13 **Andreassen H**, Hylander E, Rix M. Gender, age, and body weight are the major predictive factors for bone mineral density in Crohn's disease: a case-control cross-sectional study of 113 patients. *Am J Gastroenterol* 1999; **94**: 824-828
14 **Andreassen H**, Rix M, Brot C, Eskildsen P. Regulators of calcium homeostasis and bone mineral density in patients with Crohn's disease. *Scand J Gastroenterol* 1998; **33**: 1087-1093
15 **Silvennoinen JA**, Lehtola JK, Niemelä SE. Smoking is a risk factor for osteoporosis in women with inflammatory bowel disease. *Scand J Gastroenterol* 1996; **31**: 367-371
16 **Hyams JS**, Wyzga N, Kreutzer DL, Justinich CJ, Gronowicz GA. Alterations in bone metabolism in children with inflammatory bowel disease: an in vitro study. *J Pediatr Gastroenterol Nutr* 1997; **24**: 289-295
17 **Silvennoinen J**, Risteli L, Karttunen T, Risteli J. Increased degradation of type I collagen in patients with inflammatory bowel disease. *Gut* 1996; **38**: 223-228
18 **Robinson RJ**, Iqbal SJ, Abrams K, Al-Azzawi F, Mayberry JF. Increased bone resorption in patients with Crohn's disease. *Aliment Pharmacol Ther* 1998; **12**: 699-705
19 **Kyle J**. Crohn's disease in the northeastern and northern Isles of Scotland: an epidemiological review. *Gastroenterology* 1992; **103**: 392-399
20 **Kelly TL Sp**, von Stetton E. Performance evaluation of a multi-detector DXA device. *J Bone Miner Res* 1991; **6** Suppl 1: S168
21 **Eastell R**, Reid DM, Compston J, Cooper C, Fogelman I, Francis RM, Hosking DJ, Purdie DW, Ralston SH, Reeve J, Russell RG, Stevenson JC, Torgerson DJ. A UK Consensus Group on management of glucocorticoid-induced osteoporosis: an update. *J Intern Med* 1998; **244**: 271-292
22 **Ghosh S**, Cowen S, Hannan WJ, Ferguson A. Low bone mineral density in Crohn's disease, but not in ulcerative colitis, at diagnosis. *Gastroenterology* 1994; **107**: 1031-1039
23 **Scharla SH**, Minne HW, Lempert UG, Leidig G, Hauber M, Raedsch R, Ziegler R. Bone mineral density and calcium regulating hormones in patients with inflammatory bowel disease (Crohn's disease and ulcerative colitis). *Exp Clin Endocrinol* 1994; **102**: 44-49
24 **Vestergaard P**, Krogh K, Rejnmark L, Laurberg S, Mosekilde L. Fracture risk is increased in Crohn's disease, but not in ulcerative colitis. *Gut* 2000; **46**: 176-181
25 **Edelstein SL**, Barrett-Connor E. Relation between body size and bone mineral density in elderly men and women. *Am J Epidemiol* 1993; **138**: 160-169
26 **Felson DT**, Zhang Y, Hannan MT, Anderson JJ. Effects of weight and body mass index on bone mineral density in men and women: the Framingham study. *J Bone Miner Res* 1993; **8**: 567-573
27 **Robinson RJ**, Iqbal SJ, Al-Azzawi F, Abrams K, Mayberry JF. Sex hormone status and bone metabolism in men with Crohn's

disease. *Aliment Pharmacol Ther* 1998; **12**: 21-25
28 **Staun M**, Tjellesen L, Thale M, Schaadt O, Jarnum S. Bone mineral content in patients with Crohn's disease. A longitudinal study in patients with bowel resections. *Scand J Gastroenterol* 1997; **32**: 226-232
29 **Schulte C**, Dignass AU, Mann K, Goebell H. Bone loss in patients with inflammatory bowel disease is less than expected: a follow-up study. *Scand J Gastroenterol* 1999; **34**: 696-702
30 **Croucher PI**, Vedi S, Motley RJ, Garrahan NJ, Stanton MR, Compston JE. Reduced bone formation in patients with osteoporosis associated with inflammatory bowel disease. *Osteoporos Int* 1993; **3**: 236-241
31 **Hansen MA**, Overgaard K, Riis BJ, Christiansen C. Role of peak bone mass and bone loss in postmenopausal osteoporosis: 12 year study. *BMJ* 1991; **303**: 961-964
32 **Uebelhart D**, Schlemmer A, Johansen JS, Gineyts E, Christiansen C, Delmas PD. Effect of menopause and hormone replacement therapy on the urinary excretion of pyridinium cross-links. *J Clin Endocrinol Metab* 1991; **72**: 367-373
33 **Garnero P**, Shih WJ, Gineyts E, Karpf DB, Delmas PD. Comparison of new biochemical markers of bone turnover in late postmenopausal osteoporotic women in response to alendronate treatment. *J Clin Endocrinol Metab* 1994; **79**: 1693-1700
34 **Chesnut CH 3rd**, Bell NH, Clark GS, Drinkwater BL, English SC, Johnson CC Jr, Notelovitz M, Rosen C, Cain DF, Flessland KA, Mallinak NJ. Hormone replacement therapy in postmenopausal women: urinary N-telopeptide of type I collagen monitors therapeutic effect and predicts response of bone mineral density. *Am J Med* 1997; **102**: 29-37
35 **Dresner-Pollak R**, Karmeli F, Eliakim R, Ackerman Z, Rachmilewitz D. Increased urinary N-telopeptide cross-linked type 1 collagen predicts bone loss in patients with inflammatory bowel disease. *Am J Gastroenterol* 2000; **95**: 699-704
36 **Scott EM**, Gaywood I, Scott BB. Guidelines for osteoporosis in coeliac disease and inflammatory bowel disease. British Society of Gastroenterology. *Gut* 2000; **46** Suppl 1: i1-i8
37 **Gough A**, Sambrook P, Devlin J, Lilley J, Huisoon A, Betteridge J, Franklyn J, Nguyen T, Morrison N, Eisman J, Emery P. Effect of vitamin D receptor gene alleles on bone loss in early rheumatoid arthritis. *J Rheumatol* 1998; **25**: 864-868

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CLINICAL RESEARCH

Expressions of sonic hedgehog, patched, smoothened and Gli-1 in human intestinal stromal tumors and their correlation with prognosis

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Abstract

AIM: To investigate the role that the hedgehog (Hh) signaling pathway, which includes sonic hedgehog (Shh), Patched (Ptc), Smoothened (Smo) and Gli-1, plays in human gastrointestinal stromal tumors (GISTs).

METHODS: Surgically resected specimens from patients with GISTs, leiomyomas and schwannomas were examined by immunohistochemical staining for aberrant expression of hedgehog signaling components, Shh, Ptc, Smo and Gli-1, respectively.

RESULTS: In GISTs, 58.1% (18 of 31), 77.4% (24 of 31), 80.6% (25 of 31) and 58.1% (18 of 31) of the specimens stained positive for Shh, Ptc, Smo and Gli-1, respectively. In leiomyomas, 92.3% (12 of 13), 92.3% (12 of 13), 69.2% (9 of 13) and 92.3% (12 of 13) stained positive for Shh, Ptc, Smo and Gli-1, respectively. In schwannomas, 83.3% (5 of 6), 83.3% (5 of 6), 83.3% (5 of 6) and 100% (6 of 6) stained positive for Shh, Ptc, Smo and Gli-1, respectively. Immunohistochemistry revealed that the expressions of Shh and Gli-1 were significantly higher in leiomyomas than in GISTs ($P < 0.05$, respectively). Shh expression strongly correlated with the grade of tumor risk category and with tumor size ($P < 0.05$, respectively). However, the expressions of Ptc and Smo did not correlate with histopathological differentiation.

CONCLUSION: These results suggest that the Hh signaling pathway may play an important role in myogenic differentiation and the malignant potential of human intestinal stromal tumors.

Key words: Gastrointestinal stromal tumor; Leiomyoma; Schwannoma; Hedgehog; Immunohistochemistry

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal tract that occur from the esophagus to the anus, including in the omentum, mesentery and retroperitoneum[1]. Small GISTs are often detected during surgery for other conditions, gastroscopy or routine X-ray[1,2]. Some GISTs present with bleeding, perforation, pain, obstruction or a combination of these symptoms[3,4]. These tumors have a wide clinical spectrum from benign, incidentally detected nodules to malignant tumors[1] categorized into four risk groups: very low, low, intermediate and high[5]. Traditionally, primary mesenchymal spindle cell tumors of the gastrointestinal (GI) tract have been uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas, or leiomyosarcomas). Tumors with epithelioid cytologic features have been designated as leiomyoblastomas or epithelioid leiomyosarcomas[6]. Recently it has been postulated that GISTs originate from Cajal cells in the gastrointestinal tract, which are thought to be pacemaker cells that regulate intestinal motility[7,8]. Thus, GISTs differ from leiomyomas and schwannomas, which are of mesenchymal cell origin. Further, GISTs are characterized by the frequent expression of the bone marrow leukocytic progenitor cell antigen CD34[9] and c-kit proto-oncogene[8]. They also show a remarkable variability in their differentiation, and can be roughly divided into four major categories according to the phenotypic features of the tumors: smooth muscle type, neural type, combined

type and uncommitted type[10]. Although there are many studies about GISTs, their mechanisms of tumorigenesis, progression and differentiation remain unknown.

The Hedgehog (Hh) gene was initially isolated from Drosophila embryonic segments, and it controls patterning of imaginal disc-derived adult structures such as the eye, the appendage and the abdominal cuticle[11-13]. The mammalian Hh gene, Sonic hedgehog (Shh), is important in the patterning of many tissues and structures such as gastrointestinal epithelium, neurons, smooth muscle tissue, and bone[14-16]. Shh also plays a role in the development of the endoderm, mesoderm and ectoderm[17,18]. The response to the Hh signal is controlled by two transmembrane proteins, the tumor-suppressor Patched (Ptc) and the proto-oncogene Smoothened (Smo)[13]. Smo is a member of the seven transmembrane-receptor family[11] and its activity is suppressed by the twelve-span transmembrane Ptc. Hh stimulation releases this inhibition, leading to Smo activation of a transcriptional response[13]. Downstream targets of the pathway in vertebrates include Gli-1, which is associated with development of basal cell carcinomas and medulloblastomas[19].

There is ample evidence suggesting that the Hh signaling pathway is involved in tumor growth and differentiation. However, there are no studies that examine the expression of Hh pathway components in stromal tumors of the GI tract or the role of the Hh signaling pathway in the etiology of these tumors. Therefore, the purpose of this study is to investigate the expression of Hh pathway signaling proteins in intestinal stromal tumors.

MATERIALS AND METHODS

Tumor classification and selection

A total of 31 GISTs (all cases of stomach), 13 leiomyomas (5 cases of oesophagus, 6 of stomach and 2 of large intestine), and 6 schwannomas (5 cases of stomach and 1 of large intestine) were obtained from patients at Nagasaki University Hospital between 1997 and 2004. The tumor sizes of GISTs were 0.8-8.0 cm in diameter, leiomyomas were 0.1-2.5 cm, and schwannomas were 0.6-4.0 cm. In this study, GISTs were defined and selected as those tumors expressing both c-kit and CD34 surface antigens. Further, we classified smooth muscle actin expressing tumors into smooth muscle (M) type GISTs, S-100 protein expressing tumors into neurogenic (N) type GISTs, both smooth muscle actin and S-100 protein expressing tumors into committed type GISTs, and those expressing only c-kit and CD34 into uncommitted (UN) type GISTs[10]. And we classified histomorphologically tumors with epithelioid cell form into epithelioid cell (EP) type GISTs, spindle cell form into spindle cell (SP) type GISTs, both epithelioid and spindle cell form tumors into mixed (MIX) type GISTs [20].

We also categorized GISTs into four groups according to their malignant potential[5]. The number of mitoses was determined by counting 50 high-power fields (× 400) under Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined and selected as tumors expressing smooth muscle actin and not expressing c-kit and CD34. Schwannomas were defined and selected as tumors expressing S-100 protein and not expressing c-kit and CD34. Tumor identification and classification were determined by two independent pathologists (T. Nakayama and I. Sekine), and cases of questionable diagnosis were omitted from this study.

Immunohistochemical staining

Formalin-fixed paraffin-embedded tissues were cut into 4-μm sections, deparaffinized in xylene, and rehydrated in PBS. Deparaffinized sections were preincubated with normal bovine serum to prevent non-specific binding and then incubated overnight at 4℃ with an optimal dilution (0.1 mg/L) of a primary polyclonal goat antibody against human Shh (N-19), Ptc (C-20), Smo (N-19) and Gli-1 (C-18). Each antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The slides for Shh, Ptc, Smo and Gli-1 were then sequentially incubated with an alkaline phosphatase-conjugated donkey anti-goat immunoglobulin antibody, and the reaction products were visualized using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; BRL, Gaithersburg, MD, USA). Primary antibodies preabsorbed with excess antigen peptides or recombinant protein were used as negative controls. Basal cell carcinoma tissue served as the internal positive control for Shh, Ptc, Smo and Gli-1 immunoreactivity. Immunohistochemical analyses were performed independently by two investigators (T. Nakayama and A. Yoshizaki). Shh, Ptc, Smo and Gli-1 expression was classified into two categories depending on the percentage of cells stained: -, 0%-15% positive tumor cells; +, > 15% positive tumor cells.

Statistical analysis

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analyses. Analyses comparing the levels of Shh, Ptc, Smo and Gli-1 expression were performed using the Mann-Whitney, Kruskal-Wallis and Spearman's tests. $P < 0.05$ was taken as significant.

RESULTS

The results from the immunohistochemical analysis of Hh pathway components in human intestinal stromal tumors are summarized in Table 1. Shh expression was heterogenous and localized to the cytoplasm of cells in GISTs (Figure 1A). Ptc and Smo were localized to the cytoplasm and cell membrane, and Gli-1 was localized to the cytoplasm and nucleus of GIST tumor cells (Figure 1B-D). Immunohistochemical stainings of Hh pathway components in leiomyomas and schwannomas are shown in Figures 2 and 3, respectively. The four proteins of the Hh pathway that we examined showed patterns of expression similar to that observed in GISTs. While only 58.1% (18 of 31) of the GISTs were positive for Shh, nearly all leiomyomas and schwannomas were positive (92.3%, 12 of 13, $P < 0.05$). The following results were observed for the other proteins: 77.4% (24 of 31), 92.3% (12 of 13), 83.3% (5 of 6) of the GISTs, leiomyomas

Table 1 Aberrant expression of Hedgehog pathway signaling proteins in intestinal stromal tumors *n* (%)

			Shh		Ptc		Smo		Gli-1	
		n	+	-	+	-	+	-	+	-
GIST		31	18 (58.1)	13 (41.9)	24 (77.4)	7 (22.6)	25 (80.6)	6 (19.4)	18 (58.1)	13 (41.9)
I	GIST, M	7 (22.6)	4 (57.1)	3 (42.9)	5 (71.4)	2 (28.6)	6 (85.7)	1 (14.3)	4 (57.1)	3 (42.9)
	GIST, N	8 (25.8)	5 (62.5)	3 (37.5)	5 (62.5)	3 (37.5)	4 (50.0)	4 (50.0)	3 (37.5)	5 (62.5)
	GIST, UN	16 (51.6)	9 (56.3)	7 (43.8)	14 (87.5)	2 (12.5)	15 (93.8)	1 (6.3)[c]	11 (68.8)	5 (31.3)
II	GIST, EP	5 (16.1)	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)	3 (60.0)	2 (40.0)	2 (40.0)	3 (60.0)
	GIST, MIX	3 (9.7)	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	3 (100)	0 (0.0)	2 (66.7)	1 (33.3)
	GIST, SP	23 (74.2)	13 (56.5)	10 (43.4)	17 (73.9)	6 (26.1)	19 (82.6)	4 (17.4)	14 (60.9)	9 (39.1)
Leiomyoma		13	12 (92.3)	1 (7.7)[a]	12 (92.3)	1 (7.7)	9 (69.2)	4 (30.8)	12 (92.3)	1 (7.7)[a]
Schwannoma		6	5 (83.3)	1 (16.7)	5 (83.3)	1 (16.7)	5 (83.3)	1 (16.7)	6 (100)	0 (0.0)

n (%): Tumor cases followed by percentage (%) of total cases. [a]$P < 0.05$ between Leiomyoma and GIST in Shh or Gli-1; [c]$P < 0.05$ between GIST, UN and GIST, N.

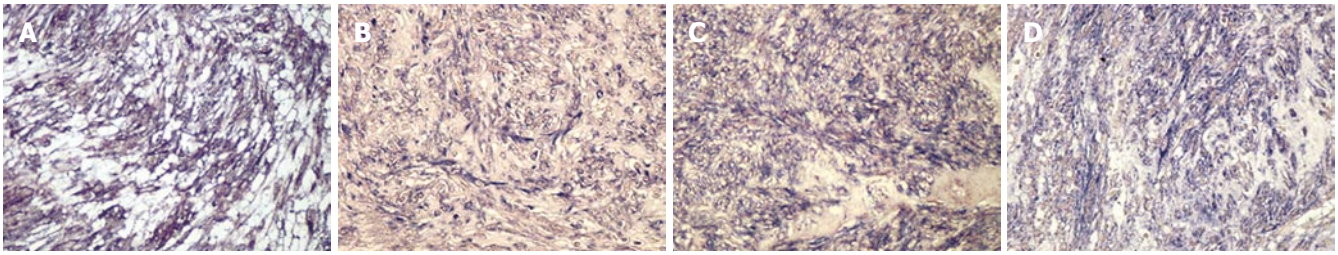

Figure 1 Immunohistochemical staining of Hh signaling components. Alkaline phosphatase reaction products demonstrating Shh (**A**), Ptc (**B**), Smo (**C**) and Gli-1 (**D**) expression. Shh is expressed in the cytoplasm, Ptc and Smo are expressed in both the cytoplasm and cell membrane, and Gli-1 is expressed in both the cytoplasm and nucleus of GIST cells (x 200).

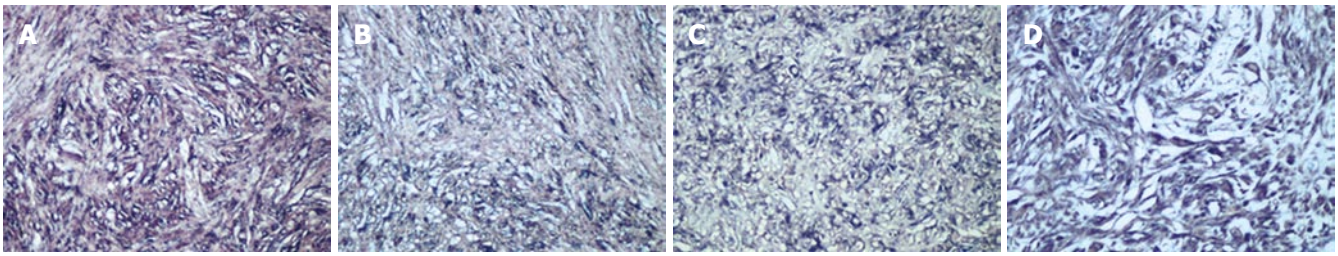

Figure 2 Immunohistochemical staining of human intestinal leiomyomas. Alkaline phosphatase reaction products demonstrating Shh (**A**), Ptc (**B**), Smo (**C**) and Gli-1 (**D**) expression (x 200).

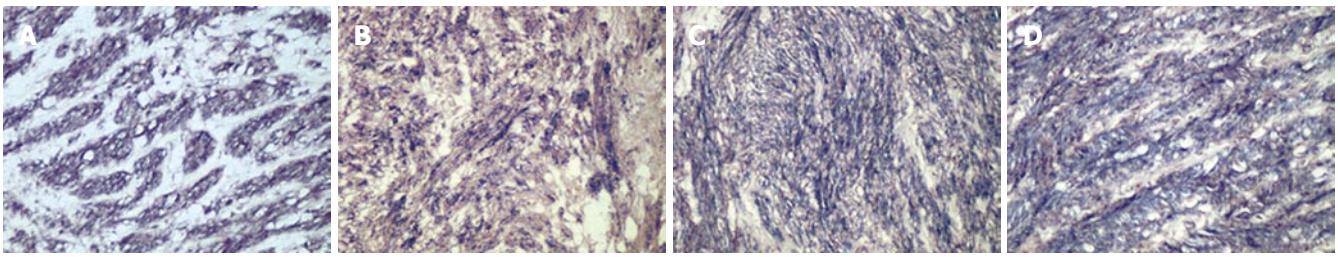

Figure 3 Immunohistochemical staining of human intestinal schwannomas. Alkaline phosphatase reaction products demonstrating Shh (**A**), Ptc (**B**), Smo (**C**) and Gli-1 (**D**) expression (x 200).

and schwannomas were positive for Ptc, respectively; 80.6% (25 of 31), 69.2% (9 of 13), 83.3% (5 of 6) of the GISTs, leiomyomas and schwannomas were positive for Smo, respectively; and 58.1% (18 of 31), 92.3% (12 of 13), 100.0% (6 of 6) of the GISTs, leiomyomas and schwannomas were positive for Gli-1, respectively. Though schwannomas expressed high levels of each protein examined, they had no statistical correlation with GISTs or leiomyomas.

Immunohistochemical analyses of Hh pathway com-

Table 2 Expression of Hedgehog pathway components in various categories of intestinal stromal tumors *n* (%)

		Shh		Ptc		Smo		Gli-1	
	n	+	-	+	-	+	-	+	-
GIST	31	18 (58.1)	13 (41.9)	24 (77.4)	7 (22.6)	25 (80.6)	6 (19.4)	18 (58.1)	13 (41.9)
Risk categories		$P < 0.05$		NS		NS		NS	
High	4	1 (25.0)	3 (75.0)	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)
Intermediate	6	3 (50.0)	3 (50.0)	5 (83.3)	1 (16.7)	4 (66.7)	2 (33.3)	2 (33.3)	4 (66.7)
Low	16	9 (56.3)	7 (43.8)	11 (68.8)	5 (31.3)	13 (81.3)	3 (18.8)	9 (56.3)	7 (43.8)
Very low	5	5 (100)	0 (0.0)	5 (100)	0 (0.0)	5 (100)	0 (0.0)	4 (80.0)	1 (20.0)
Tumor size (cm in diameter)		$P < 0.05$		NS		NS		NS	
$n \leqslant 2$	8	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)	8 (100)	0 (0.0)	6 (75.0)	2 (25.0)
$2 < n \leqslant 5$	18	10 (55.6)	8 (44.4)	14 (77.8)	4 (22.2)	13 (72.2)	5 (27.8)	8 (44.4)	10 (55.6)
$5 < n$	5	1 (20.0)	4 (80.0)	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)	4 (80.0)	1 (20.0)
Mitosis counts[1]		NS		NS		NS		NS	
0-5	23	13 (56.5)	10 (43.5)	17 (73.9)	6 (26.1)	18 (78.3)	5 (21.7)	14 (60.9)	9 (39.1)
6-10	3	2 (66.7)	1 (33.3)	3 (100)	0 (0.0)	3 (100)	0 (0.0)	2 (66.7)	1 (33.3)
11-28	5	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)	4 (80.0)	1 (20.0)	2 (40.0)	3 (60.0)

n (%): Tumor cases followed by percentage (%) of total cases; NS: Not significant; [1]Numbers per 50 areas in high-power field (× 400).

ponents in GISTs classified by cellular differentiation are shown in Table 1. In this study there was no case where a GIST was composed of combined types of cellular differentiation. The following results were observed: Shh was detected in 56.3% (9 of 16), 57.1% (4 of 7) and 62.5% (5 of 8) of UN, M and N type of GISTs; Ptc was detected in 87.5% (14 of 16), 71.4% (5 of 7) and 62.5% (5 of 8), respectively; Smo was detected in 93.8% (15 of 16), 85.7% (6 of 7) and 50.0% (4 of 8), respectively; and Gli-1 was detected in 68.8% (11 of 16), 57.1% (4 of 7) and 37.5% (3 of 8), respectively. The expression of Smo was significantly lower in UN type than in N type of GISTs ($P < 0.05$). And immunohistochemical analyses of Hh pathway components in GISTs classified histomorphologically by cellular form are shown in Table 1, also. In all GISTs, 16.1% (5 of 31) of EP cell type, 9.7% (3 of 31) of MIX cell type and 74.2% (23 of 31) of SP cell type were included, respectively. Each Hh pathway component was detected variably in different cellular type of GISTs. However, there was no correlation between the expression of Hh pathway components and cellular subtype of GISTs.

The results from immunohistochemical analysis of Hh pathway components with regard to the malignant potential of GISTs are summarized in Table 2. There were no correlations between mitosis counts and the expression levels of Hh pathway components. In contrast, the results suggested that lower levels of Shh expression correlated with lower risk GIST categories ($P < 0.05$) and smaller tumor sizes ($P < 0.05$).

DISCUSSION

Recent studies have shown that the Hh pathway plays important roles in cell differentiation, tissue patterning and embryonic development[16,19,21]. However, the role of the Hh pathway in human intestinal stromal tumors is still unclear. We investigated the expression of Shh, Ptc, Smo and Gli-1 in three types of intestinal stromal tumors (GISTs, leiomyomas and schwannomas) using immunohistochemical techniques. Our data demonstrated that Shh and Gli-1 were expressed at higher levels in leiomyomas than in GISTs. It suggested that Shh and Gli-1 expressions were correlated with myogenic differentiation. However, in the subclassification of GISTs, myogenic differentiation did not show expression of Shh or Gli-1. Moreover, the consequence of low expression of Smo in neuronal GISTs is not clear yet. Thus, future studies will address the role of the Hh pathway in the differentiation of intestinal stromal tumors.

Abrogation of the Hh pathway can also lead to tumorigenesis. In this pathway, Gli-1, which is involved in controlling cell proliferation and angiogenesis, is a key target of oncogenic action[3,13,22]. Loss of function Ptc mutations and gain of function Smo mutations are mechanisms of tumorigenesis in many types of tumors such as basal cell carcinomas, medulloblastomas, astrocytomas, small cell lung carcinomas and pancreatic cancers[19,23-25].

In this study, the expression of Shh correlated with low risk categories and small tumor sizes. It suggests that expression of Shh reduces the risk of malignant GISTs. Normally, Shh releases Smo from Ptc suppression to induce Gli-1 expression and activation[11,13,19]. Then by a negative feedback mechanism, Gli-1 suppresses the expression of Shh, which results in decrease of Gli-1. However, our data did not show a concomitant decrease in Gli-1 expression in tumors that expressed low levels of Shh. In fact, we observed high Gli-1 levels in larger tumors of high risk categories when Shh expression was low. We hypothesize that Gli-1 may be up-regulated by pathways other than the Hh pathway, or mutation of an Hh pathway component could disrupt the feedback mechanism in high risk GISTs. Thus, future studies will examine Hh pathway components in high risk GIST tumors. In conclusion, our study suggests that the Hh pathway may play important roles in myogenic differentiation and the malignant potential of human intestinal stromal tumors.

In recent studies, mutations affecting c-kit that cause constitutive tyrosine kinase activation have been shown to be important for the pathogenesis of GIST [26,27]. Joensuu

et al[28] reported a patient in whom Imatinib(STI-571, Gleevec), a tyrosine kinase inhibitor, was effective against a GIST. And Imatinib has been proven to be remarkably efficacious in heavily pretreated GISTs patients with advanced disease in phase Ⅲ clinical trials[29]. The expression of the Hh pathway is upregulated by the activation of tyrosine kinase through the epidermal growth factor pathway[30], and may be upregulated by the c-kit/ tyrosine kinase pathway.

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REFERENCES

1 **Miettinen M**, Lasota J. Gastrointestinal stromal tumors--definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. *Virchows Arch* 2001; **438**: 1-12
2 **Miettinen M**, Sarlomo-Rikala M, Sobin LH, Lasota J. Esophageal stromal tumors: a clinicopathologic, immunohistochemical, and molecular genetic study of 17 cases and comparison with esophageal leiomyomas and leiomyosarcomas. *Am J Surg Pathol* 2000; **24**: 211-222
3 **Miettinen M**, Sarlomo-Rikala M, Sobin LH, Lasota J. Gastrointestinal stromal tumors and leiomyosarcomas in the colon: a clinicopathologic, immunohistochemical, and molecular genetic study of 44 cases. *Am J Surg Pathol* 2000; **24**: 1339-1352
4 **Ueyama T**, Guo KJ, Hashimoto H, Daimaru Y, Enjoji M. A clinicopathologic and immunohistochemical study of gastrointestinal stromal tumors. *Cancer* 1992; **69**: 947-955
5 **Fletcher CD**, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: a consensus approach. *Int J Surg Pathol* 2002; **10**: 81-89
6 **Appelman HD**. Mesenchymal tumors of the gut: historical perspectives, new approaches, new results, and does it make any difference? *Monogr Pathol* 1990; (**31**): 220-246
7 **Sanders KM**. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology* 1996; **111**: 492-515
8 **Sircar K**, Hewlett BR, Huizinga JD, Chorneyko K, Berezin I, Riddell RH. Interstitial cells of Cajal as precursors of gastrointestinal stromal tumors. *Am J Surg Pathol* 1999; **23**: 377-389
9 **Miettinen M**, Virolainen M. Gastrointestinal stromal tumors--value of CD34 antigen in their identification and separation from true leiomyomas and schwannomas. *Am J Surg Pathol* 1995; **19**: 207-216
10 **Rosai J**. Stromal tumors. Rosai and Ackerman's surgical pathology. 9th ed. London: Mosby Press, 2004: 674-680
11 **Kalderon D**. Transducing the hedgehog signal. *Cell* 2000; **103**: 371-374
12 **McMahon AP**. More surprises in the Hedgehog signaling pathway. *Cell* 2000; **100**: 185-188
13 **Taipale J**, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. *Nature* 2001; **411**: 349-354
14 **Hardcastle Z**, Hui CC, Sharpe PT. The Shh signalling pathway in early tooth development. *Cell Mol Biol (Noisy-le-grand)* 1999; **45**: 567-578
15 **Lamm ML**, Catbagan WS, Laciak RJ, Barnett DH, Hebner CM, Gaffield W, Walterhouse D, Iannaccone P, Bushman W. Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. *Dev Biol* 2002; **249**: 349-366
16 **van den Brink GR**, Hardwick JC, Nielsen C, Xu C, ten Kate FJ, Glickman J, van Deventer SJ, Roberts DJ, Peppelenbosch MP. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. *Gut* 2002; **51**: 628-633
17 **Laufer E**, Nelson CE, Johnson RL, Morgan BA, Tabin C. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 1994; **79**: 993-1003
18 **Niswander L**, Jeffrey S, Martin GR, Tickle C. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 1994; **371**: 609-612
19 **Berman DM**, Karhadkar SS, Hallahan AR, Pritchard JI, Eberhart CG, Watkins DN, Chen JK, Cooper MK, Taipale J, Olson JM, Beachy PA. Medulloblastoma growth inhibition by hedgehog pathway blockade. *Science* 2002; **297**: 1559-1561
20 **Fletcher CD**, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach. *Hum Pathol* 2002; **33**: 459-465
21 **Ruiz i Altaba A**. Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. *Development* 1998; **125**: 2203-2212
22 **Saldanha G**. The Hedgehog signalling pathway and cancer. *J Pathol* 2001; **193**: 427-432
23 **Thayer SP**, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, Qi YP, Gysin S, Fernández-del Castillo C, Yajnik V, Antoniu B, McMahon M, Warshaw AL, Hebrok M. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 2003; **425**: 851-856
24 **Watkins DN**, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* 2003; **422**: 313-317
25 **Xie J**, Murone M, Luoh SM, Ryan A, Gu Q, Zhang C, Bonifas JM, Lam CW, Hynes M, Goddard A, Rosenthal A, Epstein EH Jr, de Sauvage FJ. Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* 1998; **391**: 90-92
26 **Plaat BE**, Hollema H, Molenaar WM, Torn Broers GH, Pijpe J, Mastik MF, Hoekstra HJ, van den Berg E, Scheper RJ, van der Graaf WT. Soft tissue leiomyosarcomas and malignant gastrointestinal stromal tumors: differences in clinical outcome and expression of multidrug resistance proteins. *J Clin Oncol* 2000; **18**: 3211-3220
27 **Van Glabbeke M**, van Oosterom AT, Oosterhuis JW, Mouridsen H, Crowther D, Somers R, Verweij J, Santoro A, Buesa J, Tursz T. Prognostic factors for the outcome of chemotherapy in advanced soft tissue sarcoma: an analysis of 2,185 patients treated with anthracycline-containing first-line regimens--a European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group Study. *J Clin Oncol* 1999; **17**: 150-157
28 **Joensuu H**, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, Silberman S, Capdeville R, Dimitrijevic S, Druker B, Demetri GD. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N Engl J Med* 2001; **344**: 1052-1056
29 **Maki RG**. Gastrointestinal Stromal Tumors Respond to Tyrosine Kinase-targeted Therapy. *Curr Treat Options Gastroenterol* 2004; **7**: 13-17
30 **Stepan V**, Ramamoorthy S, Nitsche H, Zavros Y, Merchant JL, Todisco A. Regulation and function of the sonic hedgehog signal transduction pathway in isolated gastric parietal cells. *J Biol Chem* 2005; **280**: 15700-15708

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CLINICAL RESEARCH

Treatment of hepatitis C virus genotype 4 with peginterferon alfa-2a: Impact of bilharziasis and fibrosis stage

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Abstract

AIM: To evaluate pegylated interferon alpha2a (PegIFN-α2a) in Egyptian patients with HCV genotype 4, and the impact of pretreatment viral load, co-existent bilharziasis and histological liver changes on response rate.

METHODS: A total of 73 naïve patients (61 with history of bilharziasis) with compensated chronic HCV genotype 4 were enrolled into: group A (38 patients) who received 180 mg PegIFN-alpha2a subcutaneously once weekly for a year and group B (35 patients) received IFN alpha-2a 3 MU 3 times weekly. Ribavirin was added to each regimen at a dose of 1200 mg. Patients were followed for 72 wk and sustained response was assessed.

RESULTS: Significant improvement in both end of treatment response (ETR) ($P < 0.002$) and sustained response (SR) ($P < 0.05$) was noted with pegylated interferon, where ETR was achieved in 29 (76.3%) and 14 patients (40%) in both groups respectively, and 25 patients in group A (65.8%) and 9 (25.7%) in group B could retain negative viraemia by the end of follow up period. Sustained virological response (SVR) showed a significant negative correlation with age and positive correlation with pretreatment inflammation in patients receiving PegIFN. Viral clearance after 3 mo of therapy was associated with high incidence of ETR and SR ($P < 0.001$), but without significant difference between both forms of interferon. Significant improvement in response was achieved in patients with high grade fibrosis (grade 3 and 4) with PegIFN-α2a, where SR was seen in 5 out of 13 patients in group A, but none in group B. There was no significant difference in response between bilharzial and non-bilharzial patients in both groups. In terms of safety and tolerability, neutropenia was the predominant side effect; both drugs were comparable.

CONCLUSION: PegIFN-α2a combined with ribavirin results in improvement in sustained response in HCV genotype 4, irrespective of history of bilharzial infestation.

Key words: Hepatitis C virus; Genotype 4; Pegasys; Bilharziasis

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INTRODUCTION

Hepatitis C is comparable to a 'viral time bomb'. The WHO estimates that about 200 million people, 3% of the world's population, are infected with hepatitis C virus (HCV) and 3 to 4 million persons are newly infected each year. The striking genetic heterogeneity of RNA genome of HCV is well recognized. Six major genotypes and over 50 subtypes and minor variants referred to as "quasispecies" are described[1]. HCV genotype differences seem to be of considerable clinical significance because they affect the responses to antiviral therapy[2]. HCV genotype 4 appears to be prevalent in the Middle East and Central Africa, where almost 13% of HCV carriers around the world live in the Eastern Mediterranean region. Prevalence rates of HCV genotype 4 ranges from 60% in Saudi Arabia to 90% in Egypt where it has been reported to be frequently associated with cirrhosis and a poor response to interferon (IFN)[3,4].

Concurrent HCV-genotype 4 infection and schistosomiasis result in a much more severe liver disease than that seen with either disease alone. Luckily, the activity of HCV infection seems to be partially suppressed in such patients[5]. The effect of such co-infection on hepatic fibrosis and in turn on response to treatment in HCV patients is however, conflicting. While Helal *et al* in 1989[6] and Shiha *et al* in 2002[7] reported a lack of enhancement

of hepatic pathology in the schistosomal patients, Hassan *et al* in 2002[8] suggested that schistosomiasis is an important risk factor involved in enhancement of nitric oxide levels and virus replication, which in turn may aggravate liver cell injury and hence the development of cirrhosis.

It has been reported that treatment with conventional IFN is less effective in patients with genotypes 1 and 4 than in patients with genotypes 2 and 3[9]. The high rate of HCV turnover coupled with the short half–life of the drug, limits the efficacy of conventional IFN therapy[10]. Pegylated IFN-α2a [Peg-IFN-α2a (40 kDa); Pegasys, Hoffmann-La Roche] is produced by attachment of a 40 kDa branched polyethylene glycol moiety to IFN-α2a by a stable amide bond. It is characterized by prolonged absorption half-life, restricted volume distribution, and decreased clearance compared to standard interferon, which thus increase its therapeutic efficacy with less frequent doses[11]. Recent clinical trials have shown that the response to pegylated interferon α2a plus ribavirin (RBV) therapy for chronic HCV infection is superior to that achieved with standard interferon α2a plus ribavirin therapy or peginteferon-α2a alone with a rapid decline in viral load in the first 12 wk, for all HCV genotypes[12]. Hematological adverse effects in the form of anaemia, neutropenia and thrombocytopenia are the primary laboratory abnormalities experienced during IFN plus RBV combination therapy and may necessitate dose modification and thus potentially impact outcome. This anemia is attributed to both ribavirin dose-dependent hemolysis and direct suppressive effect of interferon on erythropoiesis[13]. Hematopoietic growth factors may be useful in the management of these side effects.

The purpose of this prospective analysis is to compare the effectiveness and safety of Pegasys (40 kDa) IFN-α2a once weekly with IFN-α2a, in compensated HCV genotype 4, in combination with ribavirin. The effect of pretreatment viral load, histological liver changes and schistosomiasis co-infection on response to treatment is also assessed.

MATERIALS AND METHODS

Patients

Adult patients with chronic active hepatitis C as evidenced by positive serological test for HCV-Ab using enzyme linked immunosorbent assay (ELISA) (Ortho Diagnostics, Neckargmun, Germany), detectable serum HCV-RNA by RT-PCR (Amplicor Molecular System, Hoffmann-La Roche, Basel, Switzerland), elevated serum alanine transaminases (ALT) activity more than twice the normal value and histopathological criteria of chronic active hepatitis. Liver histology was classified according to Scheuer score system from 0-4 for both grades (necroinflammation) and stage (degree of fibrosis). Hepatocellular carcinoma was excluded by testing of α-fetoprotein and by ultrasound scanning. None of the female patients was pregnant as evidenced by negative serum pregnancy test. Breast feeders were excluded. All patients had normal serum direct and indirect bilirubin, albumin and creatinine. All patients were genotype 4 detected by the Inno LiPA HCV Ⅱ assay (Innogenetics Inc., GA, USA). Patients were excluded if co-infected with HBV, HIV. Hemochromatosis, Wilson disease or other causes for chronic liver disease were also ruled out. Other exclusion criteria included neutrophil count < 1.5×10^9/L, platelet count < 90×10^9/L or haemoglobin (Hb) < 100 g/L for female and < 110 g/L for male, positive auto-antibodies including antinuclear antibody (ANA), antimitochondrial antibody (AMA), anti-smooth muscle autoantibody (ASMA), patients with a history of severe psychiatric disease, seizure disorder, organ transplantation, or severe cardiac or pulmonary disease. All patients had normal thyroid function prior to the study and all were either non-diabetics or with controlled blood glucose level with hemoglobin A_{1C} < 8.5%. Patients were excluded if they had clinically significant retinal abnormalities, clinical gout, were a substance abuser (alcohol or I.V. drugs) or showed any medical condition requiring systemic steroids.

Safety assessment

Patients were reviewed in the Hepatology Outpatient Clinic weekly during the first month and monthly thereafter along the course of therapy to check for safety, and then followed for at least 6 mo after discontinuation of treatment to assess for sustained response. Epoetin beta (Recormon®. Roche) at a dose of 4000 U/weekly for 2 wk was given when Hb level decrease > 30 mg/L or > 25% from baseline levels. Also Filgrastim (Neupogen®. Amgen. Inc. F. Hoffmann-La Roche Ltd. Basel) 5 μg/kg was given once or twice weekly if neutrophils < 0.7×10^3/μL, while drug was discontinued completely for any patient showing Hb level < 85 g/L, neutrophils < 0.5×10^9/L, platelet < 50×10^9/L, abnormal thyroid function tests, creatinine > 177 μmol/L or ALT/AST double baseline levels. Patients requiring modification of more than 4 doses were excluded.

Efficacy assessment

The primary efficacy end point was sustained response (SR), defined as undetectable HCV RNA and normal ALT level at the end of follow up (24 wk after discontinuation of treatment). The relapse rate was calculated as percentage of patients with an end-of-treatment response in whom HCV RNA was detectable at wk 72. End of treatment response (ETR) was defined as normalization of ALT and loss of detectable serum HCV RNA at the end of treatment.

Study design

This randomized, controlled clinical trial was conducted from February 2002 to November 2004. The study consisted of a screening phase, which began 2 mo prior to the first dose of the drug under evaluation. Examination established eligibility of patients according to inclusion/exclusion criteria. After a written informed consent was obtained in accordance with the Helsinki Declaration of 1979, 80 patients were randomly assigned at a 1:1 ratio to receive either, subcutaneously, once weekly 180 μg of peginterferon-α2a (Pegasys, Hoffmann-LaRoche) or IFN α2a (Roferon®, Hoffmann-LaRoche) 3 MU 3 times. Ribavirin 1200 mg at a daily oral dose was added to both

Table 1 Demographic data of the hepatitis C patients

Variable	Group A (PEG-IFN + RBV) n = 38	Group B (IFN + RBV) n = 35	P
Age (yr) (mean ± SD)	45.5 ± 6.1	45.4 ± 5.8	NS[1]
Gender			
Male	31 (81.6)	33 (94.3)	
Female	7 (18.4)	2 (5.7)	
Body mass (mean ± SD)			
Before treatment	81.9 ± 12.0	73.6 ± 7.7	0.001
After Treatment	78.9 ± 12.5	71.1 ± 7.4	0.002
Weight reduction	2.9 ± 4.3	2.6 ± 2.7	NS[1]
Inflammation stage			
0-1	7 (18.4)	8 (22.9)	NS[1]
2-3	31 (81.6)	27 (77.1)	
Grade of fibrosis			
Mild (0-2)	25 (65.8)	23 (65.7)	NS[1]
Severe (3-4)	13 (34.2)	12 (34.3)	
Bilharzial co-infection No.	31 (81.6)	30 (5.7)	

[1]Not significant.

Table 2 Comparison of response of hepatitis C in both groups

Variable	Group A (PEG-IFN + RBV) n = 38	Group B (IFN + RBV) n = 35	P
After 48 wk			
Responders	29 (76.3)	14 (40.0)	< 0.002
Non-responders	9 (23.7)	21 (60.0)	
After 72 wk			
Among responders			
Sustained response	25 (86.2)	9 (64.3)	0.124
Relapser	4 (13.8)	5 (35.7)	

regimens. Throughout the study, patients were monitored for vital signs, weight, adverse events, medication compliance, thyroid function, haematologic parameters, blood chemistry and serum HCV- RNA levels.

Statistical analysis

The data were coded, and processed on an IBM-PC compatible computer using Statistical Packages for Social Sciences (SPSS). Data were expressed as mean and standard deviation (SD) unless otherwise stated. Student-t-test was used to ascertain the significance of difference between mean values of two continuous variables and Mann-Whitney test was used for non-parametric distribution. Chi-Square analysis was performed to test for differences in proportions of categorical variables between 2 or more groups. In 2 × 2 tables, the Fisher exact test (two-tailed) was used instead of Chi-Square, in particular, when sample size was small. One-way analysis of variance (ANOVA) and non-parametric Kruskal Wallis one-way analysis of variance (ANOVA) was employed for comparison of several group means and to determine the presence of significant differences between group means. The Pearson's correlation coefficient was used to evaluate the strength association between two variables. The level $P < 0.05$ was considered as the cut-off value for significance. Multivariate logistic regression analysis was performed.

RESULTS

Seventy-three patients out of 80 completed the study and follow up periods, and were classified into 2 groups: 38 patients received pegylated IFN and ribavirin (group A) and 35 received non-pegylated IFN and ribavirin (group B). Seven patients (2 in group A and 5 in group B) could not continue the study because of severe side effects or intolerability to treatment. Thyroid dysfunction in one patient in each group, intolerability of the drug's side effect in another one in group A and in 2 patients from group B, in addition to increase of transaminases and thrombocytopenia in 2 patients with cirrhosis in group B were the causes of drug discontinuation. Male gender was predominant in both groups, 31 and 33 respectively. Baseline demographic data and disease characteristics were similar in both groups (Table 1). Thirty-one patients in group A and 30 in group B had a history of bilharziasis treated with either tarter emetic (44 patients) or praziquentel (17 patients). Among these, one had histological pattern of bilharzial granuloma in liver tissue, but none had active bilharziasis prior to treatment.

Patients who received pegylated IFN (29, 76.3%) showed a significant ETR in comparison to those receiving non-peg-IFN (14, 40%) ($P < 0.002$). A significant ($P < 0.05$) improvement in SR was noticed with Peg-IFN, where 25 patients in group A (65.8% of total number of patients who completed the study) and 9 (25.7 %) in group B could retain negative viraemia at the end of follow up period (Table 2). A significant negative correlation between age and sustained response was noted in both groups ($P = 0.015$) without a significant difference between both drugs. There was no correlation between gender, pre-treatment, viraemia or body weight and response rate (Table 3). There was a significant positive correlation between pre-treatment ALT and ETR in patients receiving Peg-IFN but not in patients receiving IFN. Also, a significant positive correlation ($P < 0.05$) was found between stage of hepatic inflammation and response rate in patients treated with peg-IFN, but not with IFN (Table 3). Intent to treat (ITT) analysis showed significant improvement in both of ETR and SR with peg-IFN therapy, where ETR was 72.5% and 35%, respectively, while SR was 62.5% and 22.5% ($P < 0.002$).

Regarding viraemia, there was no significant difference between responders and non-responders in both groups and within the same group. Viral clearance after 12 wk of therapy was associated with high incidence of ETR and SR ($P < 0.001$), but also, without significant difference between both groups. By studying the relation between histopathological activity and response, treatment with peg-IFN showed a significant improvement in response and sustained response in patients with severe fibrosis (grade 3 and 4). Only one patient out of 11 with severe fibrosis showed ETR with conventional IFN therapy and unfortunately relapsed after discontinuation of treatment, while 8 patients out of 13 showed ETR with peg-IFN therapy and 5 of them could retain negative viremia at

Table 3 Comparison between responders and non-responders in both hepatitis C groups (mean ± SD)

Variable	Group A (PEG-IFN+RBV) *n* = 38			Group B (IFN+RBV) *n* = 35		
	R[1]	NR[2]	SR[3]	R[1]	NR[2]	SR[3]
Age (yr)	44.7 ± 5.4	47.8 ± 7.9	44.2 ± 5.8	45.23 ± 5.2	45.6 ± 6.2	42.9 ± 4.6
Body mass (kg)	82.6 ± 10.2	79.7 ± 17.0	82.7 ± 9.8	74.6 ± 9.5	73.1 ± 6.6	77.3 ± 9.6
ALT (μkat/L)						
Before treatment	2.559 ± 1.564	1.524 ± 0.082	2.757 ± 1.607	1.494 ± 0.432	1.810 ± 0.570	1.524 ± 0.515
After 3 mo	1.065 ± 0.980	1.095 ± 0.767	1.084 ± 1.039	0.405 ± 0.112	1.135 ± 0.741	0.487 ± 0.263
Baseline HCV RNA (MU/L)	473 934 ± 373 542	496 256 ± 667 356	472 444 ± 402 109	312 786 ± 185 583	361 857 ± 339 942	346 667 ± 207 364

[1]Responders; [2]Non responder; [3]Sustained response.

Table 4 Comparison of response in both hepatitis C groups according to histopathological changes *n* (%)

Variable	*n*	Responders	Non responders	Among responders	
				Sustained response	Relapse
Severe Fibrosis					
A[1]	13	8 (61.5)	5 (38.5)	5 (62.5)	3 (37.5)
B[2]	11	1 (9.1)	10 (90.9)	0 (0.0)	1 (100.0)
Mild fibrosis					
A[1]	25	21 (84.0)	4 (15.0)	20 (95.2)	1 (4.8)
B[2]	24	13 (54.2)	11 (45.8)	9 (69.2)	4 (30.8)
Severe inflammation					
A[1]	31	22 (71.0)	9 (29.0)	18 (81.8)	4 (18.2)
B[2]	27	7 (25.9)	20 (74.1)	4 (57.1)	3 (42.9)
Mild inflammation					
A[1]	7	7 (100.0)	0 (0.0)	7 (100.0)	0 (0.0)
B[2]	8	7 (87.5)	1 (12.5)	5 (71.4)	2 (28.6)

[1]Group A = PEG-IFN+RBV; [2]Group B = IFN + RBV.

72 wk (Table 4). There was no significant difference in response in bilharzial and non-bilharzial patients in both groups, where SR was achieved in 27 patients co-infected with bilharziasis (60.7%) and in 7 cases of HCV alone (58.3%) (Table 5).

With respect to safety and tolerability, peg-IFN was comparable to conventional IFN. Weight reduction was similar in both groups, where the mean reduction was 2.9 ± 4.3 kg and 2.6 ± 2.7 kg respectively. After flu-like picture, hematological side effects represented the commonest encountered problem (Table 6). Although anemia was seen in 71.1% and 65.7% in both groups respectively, only 39.5% and 37.1% in both groups developed Hb drop more than 30 mg/L or > 30% of baseline level and required growth stimulating factors (These figures were related only to those patients who completed the study). Two patients from group B with cirrhotic changes were withdrawn because of thrombocytopenia. The proportions of patients withdrawn from treatment because of laboratory abnormalities or other adverse effects were similar in both groups and no new or unexpected adverse effects specific to peginterferon were presented. On multivariate logistic regression analysis, no significant predictive values were noted.

DISCUSSION

Hepatitis genotype is now recognized as the most important baseline characteristic determining treatment regimen and the most useful predictor of response[14]. Slow viral dynamics, particularly second-phase decay[15] and limited effectiveness of IFN in blocking the virion have been implicated in poor response to IFN therapy in hepatitis C genotype 4 patients[16], which genotype has been described as a difficult-to-treat one. Unfortunately, this is the predominant genotype in the Middle East where large numbers of affected individuals are reported. A great improvement in response has recently been noted in all genotypes after the introduction of pegylated forms of IFN, whether as monotherapy or combined with ribavirin. Data presented in this study further reinforce the superior efficacy of PegIFN/Ribavirin combination therapy in terms of ETR and SVR in HCV genotype 4 infection. This is in accordance with earlier reports (Thakeb *et al* 2004[17]; Shobokshi *et al* 2004[18]; and Diago *et al* 2004[19]). The lower SR noted compared to that reported by Diago *et al*[19] for western genotype 4 cases (65.8% *vs* 79%) may reflect a difference in sensitivity of genotype 4 subtypes to PegIFN-α2a. The improved response to PegIFN-α2a in genotype 4 compared to conventional IFN and PegIFN-α2a previously reported by us[20,21] can be attributed, at least in part, to a high and persistent trough serum level of PegIFN-α2a in the first 4 wk of treatment which led to rapid viral eradication[22,23], or the recently reported third phase decay with PegIFN-α2a in the first 1-4 wk of therapy[24]. Other causes also include

Table 5 Comparison between responders and non-responders in both hepatitis C groups

Variable	Responders n = 43	Non Responders n = 30	P
Gender			
Male	38 (88.4)	26 (86.7)	NS[1]
Female	5 (11.6)	4 (13.3)	
Body mass (kg, Mean ± SD)			
Before treatment	80.1 ± 10.6	75.1 ± 10.9	NS[1]
After treatment	76.9 ± 10.6	72.9 ± 11.5	NS[1]
ALT			
Pre treatment	141.1 ± 88.9	101.2 ± 41.0	NS[1]
12 wk	56.2 ± 55.0	67.1 ± 44.0	NS[1]
48 wk	38.1 ± 22.7	65.0 ± 30.6	NS[1]
			NS[1]
PCR (No. of viruses in BL; mean ± SD)			
Pre treatment	416 328 ± 341 106	337 321 ± 482 826	0.036
Liver fibrosis			
Grade 1	17 (39.5)	3 (10.3)	0.009
Grade 2	17 (39.5)	11 (37.9)	
Grade 3	8 (18.6)	8 (27.6)	
Grade 4	1 (2.4)	7 (24.1)	
HCV *vs* HCV and Bilharziasis			
Co-infected	37 (84.1)	24 (82.8)	NS[1]
Non Bilharzial	7 (15.9)	5 (17.2)	
Drug used			
Pegasus	29 (67.4)	9 (30.0)	0.002
Non-pegylated IFN	14 (32.6)	21 (70.0)	

[1]Not significant.

Table 6 Comparison of side effects in both hepatitis C groups

Variable	Group A	Group B	P
Flu-like symptoms	40 (100.0)	40 (100.0)	
Discontinuation of drug	0 (0)	0 (0)	NS[1]
Thyroid dysfunction	2	3	NS[1]
Discontinuation of drug	1	2	
Psychological upset and drug intolerability	1	1	NS[1]
Discontinuation of drug	1	1	
Hematological			
Anemia (30% from baseline)	13 (26.0)	12 (24.0)	NS[1]
Neutropenia (< 0.9×10^9/L)	21 (42.0)	19 (38.0)	NS[1]
Thrombocytopenia (< 50×10^9/L)	None	2 (4)	NS[1]

[1]Not significant.

the improved patients' adherence to treatment and the use of erythropoietin and G-CSF, thus overcoming anaemia and neutropenia that have commonly led to halting therapy.

The significant correlation noted in the present study between viral clearance at wk 12 and SVR, regardless of the type of IFN used, confirms a consistent relationship between the rapidity of HCV-RNA suppression and the likelihood of achieving SR[25,26]. Conversely, patients showing positive PCR at wk 12, all failed to achieve SR regardless of ETR seen in 2 of them. This suggests that a positive PCR at wk 12 in genotype 4 cases might be considered as a strong negative predictor of response.

In terms of pretreatment predictors, only patient's age showed a negative correlation with response rate. This is probably not related to age *per se*, but rather to the age of infection, or in other words, the duration of infection, since older patients are known to develop a higher rate of liver fibrosis[27]. The presence of cirrhosis has been shown to be independently associated with decreased SVR in HCV infected patients[28]. In this respect we could demonstrate a superior SR with PegIFN-α2a over non-pegylated IFN (25.7% *vs* 0%) in both advanced fibrosis and compensated cirrhosis genotype 4 patients. Similar results were reported by Heathcote *et al* in 2000[29] and Marcellin *et al* in 2004[30].

In agreement with Tsubota *et al*[31] and contrary to Picciotto *et al*[32], we could not find any correlation between pretreatment viral load and SR, which implies that HCV-RNA levels *per se* are less influential compared to the major impact of genotype that generally determines the rate of SR.

HCV patients co-infected with schistosomiasis exhibited a unique clinical, virological and histological pattern manifested by an increased incidence of viral persistence with high HCV-RNA titers and accelerated fibrosis. This may be attributed to the fact that patients with schistosomiasis have a down regulated immune response to HCV in the form of reduced IFNγ, IL-4 and IL-10 secreted by HCV-specific T cells[33]. In spite of this, we did not find any significant difference in response to either IFN forms in cases of combined infections. This might be explained by the recent observation of El Rafei and colleagues[34] that Schistosoma mansoni by targeting a specific subset of memory CD8 cells, reduces the late differentiated memory T cell population in HCV co-infected individuals. This implies that patients infected with the genotype 4 can still mount HCV-specific T cell responses, despite the prevalence of concomitant schistosomiasis.

As for safety and tolerability, both IFN forms were comparable. As in previous reports, anemia and thrombocytopenia were the commonest haematological adverse events of the combination therapy[35]. Nevertheless, none of our patients experienced bleeding tendency or uncontrolled infection throughout the study period. The use of Epoietin α and G-CSF helped improve patients' adherence to treatment, and minimize dose reduction and discontinuation of treatment in the first 12 wk. Adherence to therapy is increasingly recognized as a key determinant in the outcome of antiviral therapy in chronic hepatitis[36] and although erythropoietin stimulates both erythropoiesis and thrombopoiesis[37], the latter effect was not demonstrated in our patients and 4 had to discontinue treatment because of thrombocytopenia.

We can conclude that concomitant HCV-genotype 4 and bilharzial infections do not seem to affect the improved responses achieved with pegylated interferon α2a plus ribavirin combination therapy. Also, in spite of the improved response in advanced fibrosis and compensated cirrhosis, advanced histopathological changes, coupled with positive viremia after 12 wk of

therapy, still remain the most important negative predictive factor for response in genotype 4 patients. A non-stop and extensive work is still needed to win the battle against HCV. Each new pharmacological modification carries with it more hope for better control of this complicated disease and tells that the difficult to treat genotype 4 will eventually be conquered.

REFERENCES

1 **Farci P**, Purcell RH. Clinical significance of hepatitis C virus genotypes and quasispecies. *Semin Liver Dis* 2000; **20**: 103-126
2 **Lyra AC**, Ramrakhiani S, Bacon BR, Di Bisceglie AM. Infection with hepatitis C virus genotype 4 in the United States. *J Clin Gastroenterol* 2004; **38**: 68-71
3 **el-Zayadi A**, Simmonds P, Dabbous H, Prescott L, Selim O, Ahdy A. Response to interferon-alpha of Egyptian patients infected with hepatitis C virus genotype 4. *J Viral Hepat* 1996; **3**: 261-264
4 **Shobokshi O**, Serebour FR, Skakni L, Al Jaser N, Tantawe AO, Sabah A, Dinish T, Qatani T, Sandokji A, Al-Blowi A, Karawi M, Al-Kayyal B, Al-Momen S, Akbar H, Ayoola A, El-Hazmi M, El-Hazmi M, Humaida A, Eissa H, Al-Quaiz M, Khawajah F, Al-Khalifa M. Peg-IFN Alfa-2a (40kda) As A Monotherapy or In Combination With Ribavirin Significantly Improve End Of Treatment Response Rate. In: HCV Genotype 4 Chronic Active Hepatitis (CAH) Patients. 53rd AASLD 2002. Boston, MA. *Hepatology* 2002; **36**: 2
5 **Gad A**, Tanaka E, Orii K, Rokuhara A, Nooman Z, Serwah AH, Shoair M, Yoshizawa K, Kiyosawa K. Relationship between hepatitis C virus infection and schistosomal liver disease: not simply an additive effect. *J Gastroenterol* 2001; **36**: 753-758
6 **Helal TE**, Danial MF, Ahmed HF. The relationship between hepatitis C virus and schistosomiasis: histopathologic evaluation of liver biopsy specimens. *Hum Pathol* 1998; **29**: 743-749
7 **Shiha G**, Zalata KR. Does schistosomiasis interfere with application of the Knodell score for assessment of chronic hepatitis C? *Med Sci Monit* 2002; **8**: CR72-CR77
8 **Hassan MI**, Kassim SK, Ali HS, Sayed el-DA, Khalifa A. Evaluation of nitric oxide (NO) levels in hepatitis C virus (HCV) infection: relationship to schistosomiasis and liver cirrhosis among Egyptian patients. *Dis Markers* 2002; **18**: 137-142
9 **Sherman M**. Response To Pegasys And Regular Interferon A-2a For Individuals With Chronic HCV And Genotype 4. Virologic Response To Pegasys With Genotype 4 In Small Study Group. AASLD November 2000. *Hepatology* 2002; **42** Suppl 1: 1A-32A
10 **Shiffman ML**. Pegylated interferons: what role will they play in the treatment of chronic hepatitis C? *Curr Gastroenterol Rep* 2001; **3**: 30-37
11 **Hadziyannis SJ**, Papatheodoridis Gv. Recent Peginteferon And Ribavirin Combination Trials. *Current Hepatitis Reports* 2004; **3**: 30-37
12 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
13 **Bräu N**. Epoetin alfa treatment for acute anaemia during interferon plus ribavirin combination therapy for chronic hepatitis C. *J Viral Hepat* 2004; **11**: 191-197
14 **Xie Y**, Xu DZ, Lu ZM, Luo KX, Jia JD, Wang YM, Zhao GZ, Zhang SL, Zhang DZ. Predictive factors for sustained response to interferon treatment in patients with chronic hepatitis C: a randomized, open, and multi-center controlled trial. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 213-219
15 **Pawlostky J**, Hezode C, Pellegrin B, Soulier A, Monder H. Early Hcv Genotype 4 Replication Kinetics During Treatment With Peginterferon Alpha-2a (Pegasys)-Ribavirin Combination. A Comparison Study with Hcv Genotype 1 And 3 Kinetics. *Hepatology* 2002; **36**: 291A
16 **Neumann AU**, Lam NP, Dahari H, Davidian M, Wiley TE, Mika BP, Perelson AS, Layden TJ. Differences in viral dynamics between genotypes 1 and 2 of hepatitis C virus. *J Infect Dis* 2000; **182**: 28-35
17 **Thakeb F**, Omar M, El Awady M, Ishak S. Randomized Controlled Trial Of Peginterferon Alfa-2a Plus Ribavirin For Chronic Hepatitis C Virus-Genotype 4 Among Egyptian Patients. Abstract. *Hepatology* 2003; **38**: 278A
18 **Shobokshi OA**, Serebour FE, Skakni L, et al. Combination therapy of peginterferon alfa-2a (40KD) (Pegasys®) and ribavirin (Copegus®) significantly enhance sustained virological and biochemical response rate in chronic hepatitis C genotype 4 patients in saudi arabia. Abstract. *Hepatology* 2003; **38**: 636A
19 **Diago M**, Hadziyannis S, Bodenheimer H, Hassanein T,Uchman S, Marcellin P, Ramadori G, Delwaide J, Sedarati F. Optimized Virological Response In Patients With Genotype 4 Chronic Hepatitis C Treated With Peginterferon Alfa-2a (40KD) (Pegasys®) In Combination With Ribavirin (RBV). Abstract. *Hepatology* 2002; **36**: 364A
20 **Derbala M**, Omar M. Efficacy of interferon therapy for hepatitis C virus in patients with schistosomiasis. results from a comparative study. *Kasr AL Aini Med J* 1998; **4** Suppl 1: 247-254
21 **Derbala M**, Amer A, Bener A, Lopez AC, Omar M, El Ghannam M. Pegylated interferon-alpha 2b-ribavirin combination in Egyptian patients with genotype 4 chronic hepatitis. *J Viral Hepat* 2005; **12**: 380-385
22 **Foster GR**. Review article: pegylated interferons: chemical and clinical differences. *Aliment Pharmacol Ther* 2004; **20**: 825-830
23 **Di bisceglie A**, Rustgi V, Thuluvath P, davis M, Ghalib R, Lyons M, Ondovik M, Lopez-Talavera J, Hamzeh F. Pharmacokinetics and pharmakodynamics of pegylated interferon-alpa2a with ribavirin in treatment naive patients with genotype 1 chronic hepatitis. 55th AASLD, 2004. Boston, MA. Abstract LB-18
24 **Herrmann E**, Lee JH, Marinos G, Modi M, Zeuzem S. Effect of ribavirin on hepatitis C viral kinetics in patients treated with pegylated interferon. *Hepatology* 2003; **37**: 1351-1358
25 **Ferenci P**, Fried MW, Shiffman ML, Smith CI, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxì A, Chaneac M, Reddy KR. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 KD)/ribavirin. *J Hepatol* 2005; **43**: 425-433
26 **Kamal SM**, El Tawil AA, Nakano T, He Q, Rasenack J, Hakam SA, Saleh WA, Ismail A, Aziz AA, Madwar MA. Peginterferon {alpha}-2b and ribavirin therapy in chronic hepatitis C genotype 4: impact of treatment duration and viral kinetics on sustained virological response. *Gut* 2005; **54**: 858-866
27 **Ticehurst J**, Hu S, Hamzeh F, Thomas D. Factors affecting hcv viral load in patients with genotype 1 infection (Abstract 411). *Hepatology* 2004; **40** Suppl: 342A
28 **Al-Faleh FZ**, Aljumah A, Rezeig M, Al-Kanawi M, Alahdal M, Al-Humayed S, Mayet I, Al-Juhani M, Al-Karawi M, George K, Sbeih F. Treatment of chronic hepatitis C genotype IV with interferon-ribavirin combination in Saudi Arabia: a multicentre study. *J Viral Hepat* 2000; **7**: 287-291
29 **Heathcote EJ**, Shiffman ML, Cooksley WG, et al. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N Engl J Med* 2000; **343**: 1673-1680
30 **Marcellin P**, Roberts S, Alberti A, Trepo C, Zeuzem S, Hoel Sette Jr, Brouwer J. Sustained virological and biochemical response to peginterferon alpha-2a plus ribavirin in patients with chronic hepatitis c and compensated cirrhosis/bridging fibrosis. *Hepatology* 2004; **40**: 531A
31 **Tsubota A**, Arase Y, Someya T, Suzuki Y, Suzuki F, Saitoh S, Ikeda K, Akuta N, Hosaka T, Kobayashi M, Kumada H. Early viral kinetics and treatment outcome in combination of high-dose interferon induction vs. pegylated interferon plus

ribavirin for naive patients infected with hepatitis C virus of genotype 1b and high viral load. *J Med Virol* 2005; **75**: 27-34

32 **Picciotto A**, Campo N, Brizzolara R, Sinelli N, Poggi G, Grasso S, Celle G. HCV-RNA levels play an important role independently of genotype in predicting response to interferon therapy. *Eur J Gastroenterol Hepatol* 1997; **9**: 67-69

33 **El-Kady IM**, Lotfy M, Badra G, El-Masry S, Waked I. Interleukin (IL)-4, IL-10, IL-18 and IFN-gamma cytokines pattern in patients with combined hepatitis C virus and Schistosoma mansoni infections. *Scand J Immunol* 2005; **61**: 87-91

34 **Elrefaei M**, El-sheikh N, Kamal K, Cao H. Analysis of T cell responses against hepatitis C virus genotype 4 in Egypt. *J Hepatol* 2004; **40**: 313-318

35 **Dieterich DT**, Spivak JL. Hematologic disorders associated with hepatitis C virus infection and their management. *Clin Infect Dis* 2003; **37**: 533-541

36 **Patel K**, Anouk T. Adherence to antiviral therapy in chronic hepatitis. *Current hepatitis reports* 2004; **3**: 10-15

37 **Homoncik MG**, Sieghart W , Formann E , Schmid M, Ferlitsch A, Ferenci P, Gangl A, Jilma B, Peck-Radosavljevic M. Erythropoietin and platelet counts, platelet function in patients with chronic hepatitis c undergoing combination antiviral therapy : a randomized, placebo-controlled, double-blind study (Abstract 527). *Hepatology* 2004; **40** suppl 1: 392A

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RAPID COMMUNICATION

Endoscopic mucosal resection of large hyperplastic polyps in 3 patients with Barrett's esophagus

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Abstract

AIM: To report the endoscopic treatment of large hyperplastic polyps of the esophagus and esophagogastric junction (EGJ) associated with Barrett's esophagus (BE) with low-grade dysplasia (LGD), by endoscopic mucosal resection (EMR).

METHODS: Cap fitted EMR (EMR-C) was performed in 3 patients with hyperplastic-inflammatory polyps (HIPs) and BE.

RESULTS: The polyps were successfully removed in the 3 patients. In two patients, with short segment BE (SSBE) ($\leqslant$ 3 cm), the metaplastic tissue was completely excised. A 2 cm circumferential EMR was performed in one patient with a polyp involving the whole EGJ. A simultaneous EMR-C of a BE-associated polypoid dysplastic lesion measuring 1 cm x 10 cm, was also carried out. In the two patients, histologic assessment detected LGD in BE. No complications occurred. Complete neosquamous re-epithelialization occurred in the two patients with SSBE. An esophageal recurrence occurred in the remaining one and was successfully retreated by EMR.

CONCLUSION: EMR-C appears to be a safe and effective method for treating benign esophageal mucosal lesions, allowing also the complete removal of SSBE.

Key words: Hyperplastic polyps; Endoscopic mucosal resection; Barrett's esophagus

INTRODUCTION

Benign esophageal tumors are rare, representing less than 1% of all esophageal tumors[1]. They are usually asymptomatic and often discovered incidentally[2]. Many classifications have been proposed for benign esophageal tumors: by histological cell types, which are divided into epithelial and non-epithelial tumors, or by the location in the esophageal wall, categorized as intramural and extramural tumors[3,4].

Epithelial polypoid lesions of the esophagus and esophagogastric junction (EGJ) are uncommon[5-8]. Among them, hyperplastic-inflammatory polyps (HIPs) often occur in combination with gastroesophageal reflux disease (GERD)[9-13]. Endoscopic mucosal resection (EMR) is a promising therapeutic option for the removal of superficial carcinomas throughout the gastrointestinal tract. This technique permits resection of the mucosa and submucosa, exposing the muscularis propria[14]. The authors report the outcome of EMR in three patients with large hyperplastic polyps of the EGJ associated with Barrett's esophagus (BE).

MATERIALS AND METHODS

Endoscopy

Upper gastrointestinal endoscopy (UGIE) was performed with standard forward-viewing videoendoscope (GIF-Q145, Olympus Optical Co. Ltd., Tokyo, Japan).

Before EMR, endoscopic ultrasonography (EUS) (GF-UMQ 130, 7.5-20 MHz, Olympus Optical Co. Ltd., Tokyo, Japan) was performed to assess lesion depth and lymph node status.

Endoscopic resection technique

EMR was performed with the "cap" technique (EMR-C). A 13 mm diameter transparent plastic cap (MH-594, Olympus Co. Ltd., Tokyo, Japan) was preloaded on the tip of a standard diagnostic forward-viewing endoscope

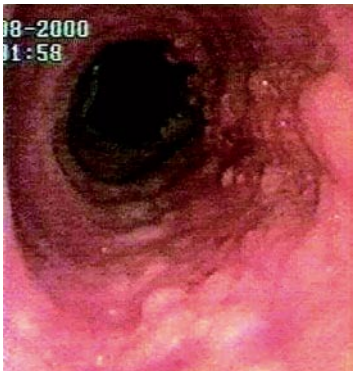

Figure 1 A sessile villous-like polyp extending for the whole length of BE.

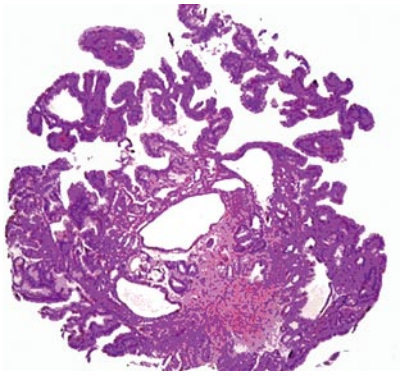

Figure 2 Hyperplastic polyp from the gastroesophageal junction was comprised of cardiac type mucosa with foveolar hyperplasia and cystic dilatation of gastric pits (HE x 100).

(GIF-Q145, Olympus Optical Co. Ltd., Tokyo, Japan). Inside the distal end of the cap is a gutter that positions the opened monofilament polypectomy snare (SD-221U-25, Olympus Co. Ltd., Tokyo, Japan). After submucosal injection of diluted epinephrine (1:200 000), with methylene blue, the cap was pressed against the mucosa and suction applied. The polypectomy snare was closed around the tissue and resection performed by endocut mode only, using the ERBE-ICC 200 cautery device (ERBE Elektromedizin Gmgh, Tubingen, Germany).

After each resection the endoscope was withdrawn to collect the tissue specimen.

Deep sedation with propofol was used to perform the endoscopic procedures.

RESULTS

All resected fragments were fixed in 40 g/L neutral formaldehyde, embedded in paraffin and serially sectioned. The sections were stained with hematoxylin and eosin; in addition all specimens were stained with periodic acid-Schiff/Alcian blue stain at pH 2.5 for evaluation of intestinal metaplasia (IM).

Immunohistochemical staining for p53 was performed in all cases (clone DO-7, BioGenex, San Ramon, CA; final dilution 1:20 000).

Case 1

A 72-year old man complained of gastroesophageal reflux symptoms since the age of 25, which became worse in the last five years, occurring also during the night. He was taking over-the-counter drugs (ranitidine) for his reflux symptoms, with poor control. He never complained of dysphagia. The past medical history of the patient was unremarkable. Endoscopy showed a 3 cm hiatal hernia, and a circumferential BE 10 cm long. A sessile villous-like polyp, 10 mm wide, extending for the whole length of BE, was observed (Figure 1). The colour of the lesion was similar to BE, and at the EGJ became wider, involving the entire circumference of the junction for a length of 15 mm.

EUS demonstrated a lesion confined to the mucosa. No pathologic lymph nodes were detected. Multiple biopsies from BE showed incomplete IM with low-grade epithelial dysplasia (LGD), rare pancreatic pits and positivity for p53; biopsies from the EGJ polyp showed proliferation of hyperplastic cardiac-type epithelium with some cystic dilatation of gastric pits, rare goblet cells, rare parietal cells (Figure 2) and some groups of cells with LGD. Additional features included oedema and inflammation in the lamina propria.

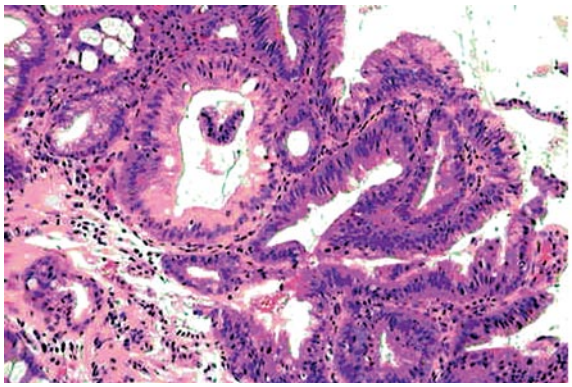

Figure 3 Sessile lesion arising in the Barrett's esophagus: the adenomatous villous-type polyp is composed of low grade dysplastic epithelium (HE x 250).

Biopsies from the sessile lesion in BE showed fragments of adenomatous villous-type with incomplete IM, epithelial LGD (Figure 3) and over expression of p53.

The patient was hospitalised and EMR-C performed. Both lesions (the long esophageal one, 1 cm × 10 cm, and its 2 cm of circumferential expansion at the EGJ), were completely removed in one endoscopic session. No intraprocedural or delayed complications occurred. On histology, the EGJ lesion was a hyperplastic polyp, and the lesion arising in BE was a villous adenomatous polyp with LGD (also named BE-associated polypoid dysplastic lesion).

Intravenous omeprazole was administered during the following 24 h. Then 40 mg of rabeprazole were given. The patient was kept fasting for 24 h. Then a liquid diet was started, and solid foods were reintroduced five days later. He was dismissed after four days on long term rabeprazole 40 mg.

UGIE was not repeated until 18 mo later, as the patient refused earlier endoscopic examinations. He was asymptomatic, and still taking 40 mg of rabeprazole. Endoscopy showed a 10 cm long area of neosquamous re-epithelialization where EMR had been performed. Three areas of mucosal abnormality (average size: 12 mm) were detected in the lower esophagus (Figure 4), and EMR was repeated, with resection of larger areas of metaplastic epithelium to favour neosquamous re-epithelialization. No

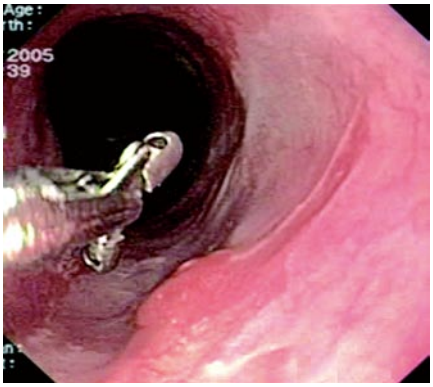

Figure 4 Areas of neosquamous re-epithelialization coexisting with tiny mucosal abnormalities.

complications occurred and the patient left the hospital three days later. Histology showed both hyperplastic cardiac-type and cystic hyperplastic gastric fundic polyps. The resected associated BE showed incomplete IM without dysplasia.

A further endoscopy performed 3 mo later, revealed large areas of re-epithelialization and no mucosal abnormalities in the residual BE. Multiple biopsies were taken from the residual BE, that showed IM without dysplasia.

Case 2

A 66-year old woman underwent endoscopy for recurrent mild epigastric pain during the previous three months, without gastroesophageal reflux symptoms. The past medical history was unremarkable. Endoscopy showed a 3 cm hiatal hernia, a short segment BE (SSBE-20 mm), and a 25 mm sessile polyp at the EGJ. Multiple biopsies showed hyperplastic squamous epithelium. EUS showed a lesion confined to the mucosa, without lymph node involvement. Biopsies of the BE showed incomplete IM with LGD, and p53 negativity.

The patient was hospitalised and EMR-C performed. The polyp and BE were both excised. No early or delayed complications occurred. The patient left the hospital three days later, and rabeprazole 40 mg was advised. The first repeat endoscopy was scheduled after 3 mo, when histology revealed proliferative hyperplastic squamous epithelium and chronic inflammatory cells in the lamina propria. Endoscopy 3 and 6 mo later showed complete neosquamous re-epithelialization of the lower esophagus, without stricture.

Case 3

A 72-year old man underwent endoscopy for recurrent epigastric pain occurring over the previous five years, without reflux symptoms. Endoscopy showed a 30 mm tongue of metaplastic epithelium in the distal esophagus, a 3 cm hiatal hernia, and a 30 mm sessile polyp at the EGJ. Biopsies from the BE and polyp showed incomplete IM and squamous epithelial hyperplasia. EUS showed a lesion confined to the mucosa. No pathologic lymph nodes were detected.

Both polyp and BE were completely removed by EMR-C. No complications occurred and the patient left the hospital three days later. He was advised to assume 40 mg of rabeprazole indefinitely. Histology confirmed that the polyp consisted of hyperplastic squamous epithelium and inflammatory infiltrate. The resected BE showed incomplete IM without dysplasia. Immunohistochemical stain for p53 was negative. Endoscopy 3 and 6 mo later showed complete neosquamous re-epithelialization of the lower esophagus, with minimal scarring.

DISCUSSION

We were able to remove large polyps of the esophagus and EGJ by EMR in 3 patients with BE, without complications. In the two patients with a SSBE, the metaplastic tissue was completely removed, allowing complete neosquamous re-epithelialization.

At histology, inflammatory polyps are characterized by hyperplastic epithelium with variable amounts of inflamed stroma[5]. Occasionally, esophageal polyps containing stroma and granulation tissue, without an epithelial component, have been reported[5-7]. Other types of polyps are the inflammatory pseudotumors, also known as inflammatory polyps with pseudomalignant erosion. They usually occur in the distal esophagus, arise from the mucosal layer and contain inflamed granulation tissue with bizarre stromal cells. Pseudomalignant erosion has been found in 14.3% of inflammatory polyps of the EGJ[15]. Inflammatory esophagogastric polyps are thought to be a complication of gastroesophageal reflux[6,16]. They have also been reported in patients with hiatal hernia (88%) and/or reflux esophagitis (91%)[6,17]. The association of hyperplastic polyps and BE is infrequent, and to our knowledge, only Abraham *et al*[5] has reported it previously. In fact, adenoma is more frequently associated with BE. It has been suggested that the use of the term "adenoma" in BE may be misleading, because it carries a "benign" connotation[18]. The appropriate term for these lesions should be "BE-associated polypoid dysplastic lesion", because they share similar clinical, pathological and molecular features as those of flat dysplasia[19].

In a series of 27 patients with a total of 30 hyperplastic esophageal and EGJ polyps, 80% of them were composed of cardiac-type mucosa, 17% of squamous mucosa, and 3% contained both cardiac and squamous mucosa. IM was present in only two polyps (7%)[5]. The location of HIPs was the EGJ region (67%), distal esophagus (30%) and mid-esophagus (3%), in accordance with other data reported in the literature[16,17].

Only four cases of hyperplastic polyps in the cervical esophagus have been reported[20-23], all arising from ectopic gastric mucosa[24,25].

Less often, polyps are located at the end of a prominent inflamed gastric fold very close to the squamocolumnar junction, also known as "the sentinel fold", seen in reflux esophagitis[26].

Histologically similar polyps have been reported in von Recklinghausen's[27], and Crohn's disease[28,29], protracted vomiting[5,30], and infectious esophagitis (Candida, Cytomegalovirus, Herpes simplex virus)[5]. Other esophageal injuries, due to drugs (K-dur, alendronate sodium and ibuprofen)[5], sclerotherapy for esophageal

varices[12], polypectomy, and photodynamic therapy[5], can result in hyperplastic polyp occurrence. Their pathogenesis is similar to that of gastric and colonic hyperplastic polyps that frequently seem to occur in response to mucosal injury[31,32]. However, there are cases in which the etiology cannot be defined[5,33]. Malignant transformation has been occasionally reported in gastric and colonic hyperplastic polyps[32,34], but not in those arising in the esophagus or EGJ.

In the study of Abraham *et al*[5], 4 of 27 patients with esophageal hyperplastic polyps also had BE. IM within cardiac-type mucosa was present in two polyps (6.7%): one contained goblet cells, and the other one was surrounded by Barrett's mucosa. Only the latter polyp (3%) showed LGD. Three out of 4 patients with BE had previous, concomitant, or subsequent development of dysplasia in the non-polypoid esophagus. Furthermore, the frequency of dysplasia within esophageal hyperplastic polyps is low and it has been reported only in those cases associated with dysplastic Barrett's esophagus[5].

In our patients, histological evaluation showed hyperplastic epithelium and inflamed stroma in the polyps located at EGJ. Histological assessment of the 10 cm long polyp arising in BE showed a "BE-associated polypoid dysplastic lesion". The treatment of HIPs of the esophagus and EGJ has changed over the years. In the past open surgical resection was considered standard care in the management of all significant esophageal lesions[2,33]. Transabdominal Nissen fundoplication operation to prevent reflux has also been used in cases where the correlation between HIPs and GERD was documented[11].

More recently thoracoscopic approaches have been performed for mucosal or submucosal lesions > 30 mm[2]. Endoscopic polypectomy for HIPs is the current treatment for lesions ⩽ 30 mm[15,16,27]. Oguma *et al*[21] reported the successful removal of a pedunculated hyperplastic polyp (size: 9.0 mm × 4.5 mm × 4.0 mm) arising in the ectopic gastric mucosa in the cervical esophagus[23]. Two of our patients had LGD in the BE. In patients with BE, cancer can develop through several steps encompassing LGD, high-grade dysplasia (HGD) and invasive adenocarcinoma[35]. However, adenocarcinomas have also been detected in patients whose previous biopsies revealed only low-grade or even no dysplasia[36-39]. The information on the natural history of LGD is limited. Moreover, the diagnosis of LGD is highly subjective and associated with interobserver variability[40]. In three articles, the reported rate of progression from LGD to HGD or adenocarcinoma was 10%[41], 12%[42], and 28%[37]. In these studies the agreement among pathologists in diagnosing LGD was associated with an increased risk of progression to HGD or cancer.

At present, patients with HGD are advised to undergo surgical treatment due to the reported frequency of undetected synchronous cancers[43,44]. However, surgery carries substantial morbidity and mortality[45]. EMR could become a therapeutic alternative to esophagectomy in selected patients[46-50].

Some authors suggest EMR in patients with BE and LGD to improve the histologic accuracy[46,51]. However, complications can occur during this procedure. Bleeding and perforation have been reported in a median 10% of patients and less than 1% of cases, respectively[50]. Esophageal stenosis is a late complication, and may occur in 0%-30% of cases[50]. Larger EMR resections may increase the risk of stenosis. In a study encompassing 137 patients, stenosis occurred only when EMR involved more than two-thirds of the esophageal circumference[52]. However, in one report of circumferential EMR, only two of 12 patients developed stenosis[53]. Overall, complications seem fewer for EMR than for surgical resection[54].

We were able to remove the large sessile lesions with EMR-C, in one session, without complications. In two of our patients, with non-circumferential SSBE, the metaplastic epithelium was completely removed. The patient with a 10 cm long BE showed a large neosquamous re-epithelialization, involving half of the BE surface. In the same patient the 20 mm circumferential EMR at the EGJ level did not cause any stricture.

In conclusion, our experience indicates that esophageal and EGJ hyperplastic polyps could represent an exuberant epithelial regeneration following mucosal injury, particularly GERD. The presence of BE in all of them seems to confirm the hypothesis of GERD in causing inflammatory reaction. The malignant transformation of these polyps is rare, but possible[5]. Also, the presence of dysplasia within a segment of BE is patchy, and random biopsies may fail to detect it[55].

We performed EMR to remove the large hyperplastic polyps and the surrounding areas of BE, as EMR provides greater diagnostic precision than endoscopic biopsy. In the study of Conio *et al*[50] reclassification of the histology after EMR occurred in 26% in a series of 39 patients with BE. Other authors have reported reclassification of the histologic diagnosis after EMR in 0% to 75% of cases[53,56]. We can conclude that EMR is minimally invasive and a low risk method in treating large benign esophageal mucosal lesions.

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REFERENCES

1 **Watson RR**, O'Connor TM, Weisel W. Solid benign tumors of the esophagus. *Ann Thorac Surg* 1967; **4**: 80-91
2 **Kinney T**, Waxman I. Treatment of benign esophageal tumors by endoscopic techniques. *Semin Thorac Cardiovasc Surg* 2003; **15**: 27-34
3 **Kessler B**, Stegemann B, Langhans P, Pircher W. [Benign tumors of the esophagus (author's transl)]. *Leber Magen Darm* 1980; **10**: 28-31
4 **Choong CK**, Meyers BF. Benign esophageal tumors: introduction, incidence, classification, and clinical features. *Semin Thorac Cardiovasc Surg* 2003; **15**: 3-8
5 **Abraham SC**, Singh VK, Yardley JH, Wu TT. Hyperplastic polyps of the esophagus and esophagogastric junction: histologic and clinicopathologic findings. *Am J Surg Pathol* 2001; **25**: 1180-1187
6 **Bleshman MH**, Banner MP, Johnson RC, DeFord JW. The inflammatory esophagogastric polyp and fold. *Radiology* 1978; **128**: 589-593
7 **Ghahremani GG**, Fisher MR, Rushovich AM. Prolapsing

inflammatory pseudopolyp-fold complex of the oesophagogastric region. *Eur J Radiol* 1984; **4**: 47-51
8 **Kato S**, Ozawa A, Shibuya H, Nakagawa H, Naganuma H. Inflammatory esophagogastric polyp and fold in an adolescent. *Acta Paediatr Jpn* 1993; **35**: 53-56
9 **Hu C**, Levine MS, Laufer I. Solitary ulcers in reflux esophagitis: radiographic findings. *Abdom Imaging* 1997; **22**: 5-7
10 **Eller JL**, Ziter FM Jr, Zuck TF, Brott W. Inflammatory polyp: a complication in esophagus lined by columnar epithelium. *Radiology* 1971; **98**: 145-146
11 **Rabin MS**, Bremner CG, Botha JR. The reflux gastroesophageal polyp. *Am J Gastroenterol* 1980; **73**: 451-452
12 **Van der Veer LD**, Kramer K, Relkin R, Clearfield H. The esophagogastric polyp-fold complex. *Am J Gastroenterol* 1984; **79**: 918-920
13 **Zitsman JL**, Schullinger JN, Berdon WE. Inflammatory esophagogastric polyps: resolution following antireflux surgery. *J Pediatr Surg* 1988; **23**: 1016-1017
14 **Inoue H**. Endoscopic mucosal resection for the entire gastrointestinal mucosal lesions. *Gastrointest Endosc Clin N Am* 2001; **11**: 459-478
15 **Moriyama T**, Matsumoto T, Jo Y, Iwai K, Yao T, Iida M. Pseudomalignant erosion in an inflammatory polyp at esophagocardial junction. *Gastrointest Endosc* 2003; **57**: 987-989
16 **Glassman M**, Bostwick HE, Godine L, Newman LJ. Endoscopic removal of inflammatory esophagogastric polyps in children. *J Pediatr Gastroenterol Nutr* 1991; **13**: 110-114
17 **Styles RA**, Gibb SP, Tarshis A, Silverman ML, Scholz FJ. Esophagogastric polyps: radiographic and endoscopic findings. *Radiology* 1985; **154**: 307-311
18 **Thurberg BL**, Duray PH, Odze RD. Polypoid dysplasia in Barrett's esophagus: a clinicopathologic, immunohistochemical, and molecular study of five cases. *Hum Pathol* 1999; **30**: 745-752
19 **Arnold GL**, Mardini HE. Barrett's esophagus-associated polypoid dysplasia: a case report and review of the literature. *Dig Dis Sci* 2002; **47**: 1897-1900
20 **Shah KK**, DeRidder PH, Shah KK. Ectopic gastric mucosa in proximal esophagus. Its clinical significance and hormonal profile. *J Clin Gastroenterol* 1986; **8**: 509-513
21 **Raine CH**. Ectopic gastric mucosa in the upper esophagus as a cause of dysphagia. *Ann Otol Rhinol Laryngol* 1983; **92**: 65-66
22 **Chatelain D**, Fléjou JF. [Hyperplastic polyp in heterotopic gastric mucosa. A rare lesion of the cervical esophagus]. *Ann Pathol* 1998; **18**: 415-417
23 **Oguma J**, Ozawa S, Omori T, Kitagawa Y, Saikawa Y, Mikami S, Kitajima M. EMR of a hyperplastic polyp arising in ectopic gastric mucosa in the cervical esophagus: case report. *Gastrointest Endosc* 2005; **61**: 335-338
24 **Takagi A**, Ema Y, Horii S, Morishita M, Miyaishi O, Kino I. Early adenocarcinoma arising from ectopic gastric mucosa in the cervical esophagus. *Gastrointest Endosc* 1995; **41**: 167-170
25 **Mion F**, Lambert R, Partensky C, Cherkaoui M, Berger F. High-grade dysplasia in an adenoma of the upper esophagus developing on heterotopic gastric mucosa. *Endoscopy* 1996; **28**: 633-635
26 **Bach KK**, Postma GN, Koufman JA. Esophagitis with an inflammatory polyp. *Ear Nose Throat J* 2002; **81**: 824
27 **De Giacomo C**, Gullotta R, Perotti P, Bawa P, Cornaggia M, Fiocca R. Hyperplastic esophagogastric polyps in two children with neurofibromatosis type 1. *J Pediatr Gastroenterol Nutr* 1994; **18**: 107-110
28 **Shim KS**, Suh JM, Baeg NJ, Yang YS, Kim BS. Post-inflammatory polyps of esophagus: a rare sequela of endoscopic injection sclerotherapy for esophageal varix. *Gastrointest Endosc* 1993; **39**: 861-862
29 **Cockey BM**, Jones B, Bayless TM, Shauer AB. Filiform polyps of the esophagus with inflammatory bowel disease. *AJR Am J Roentgenol* 1985; **144**: 1207-1208
30 **Walker RS**, Breuer RI, Victor T, Gore RM. Crohn's esophagitis: a unique cause of esophageal polyposis. *Gastrointest Endosc* 1996; **43**: 511-515
31 **Abraham SC**, Singh VK, Yardley JH, Wu TT. Hyperplastic polyps of the stomach: associations with histologic patterns of gastritis and gastric atrophy. *Am J Surg Pathol* 2001; **25**: 500-507
32 **Jørgensen H**, Mogensen AM, Svendsen LB. Hyperplastic polyposis of the large bowel. Three cases and a review of the literature. *Scand J Gastroenterol* 1996; **31**: 825-830
33 **Croyle P**, Nikaidoh H, Currarino G. Inflammatory esophagogastric junction polyp. *Am J Gastroenterol* 1981; **76**: 438-440
34 **Morimoto LM**, Newcomb PA, Ulrich CM, Bostick RM, Lais CJ, Potter JD. Risk factors for hyperplastic and adenomatous polyps: evidence for malignant potential? *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 1012-1018
35 **van Sandick JW**, van Lanschot JJ, Kuiken BW, Tytgat GN, Offerhaus GJ, Obertop H. Impact of endoscopic biopsy surveillance of Barrett's oesophagus on pathological stage and clinical outcome of Barrett's carcinoma. *Gut* 1998; **43**: 216-222
36 **Spechler SJ**. Dysplasia in Barrett's esophagus: limitations of current management strategies. *Am J Gastroenterol* 2005; **100**: 927-935
37 **Skacel M**, Petras RE, Gramlich TL, Sigel JE, Richter JE, Goldblum JR. The diagnosis of low-grade dysplasia in Barrett's esophagus and its implications for disease progression. *Am J Gastroenterol* 2000; **95**: 3383-3387
38 **Schnell TG**, Sontag SJ, Chejfec G, Aranha G, Metz A, O' Connell S, Seidel UJ, Sonnenberg A. Long-term nonsurgical management of Barrett's esophagus with high-grade dysplasia. *Gastroenterology* 2001; **120**: 1607-1619
39 **O'Connor JB**, Falk GW, Richter JE. The incidence of adenocarcinoma and dysplasia in Barrett's esophagus: report on the Cleveland Clinic Barrett's Esophagus Registry. *Am J Gastroenterol* 1999; **94**: 2037-2042
40 **Sharma P**. Low-grade dysplasia in Barrett's esophagus. *Gastroenterology* 2004; **127**: 1233-1238
41 **Weston AP**, Banerjee SK, Sharma P, Tran TM, Richards R, Cherian R. p53 protein overexpression in low grade dysplasia (LGD) in Barrett's esophagus: immunohistochemical marker predictive of progression. *Am J Gastroenterol* 2001; **96**: 1355-1362
42 **Reid BJ**, Levine DS, Longton G, Blount PL, Rabinovitch PS. Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. *Am J Gastroenterol* 2000; **95**: 1669-1676
43 **Collard JM**. High-grade dysplasia in Barrett's esophagus. The case for esophagectomy. *Chest Surg Clin N Am* 2002; **12**: 77-92
44 **Heitmiller RF**, Redmond M, Hamilton SR. Barrett's esophagus with high-grade dysplasia. An indication for prophylactic esophagectomy. *Ann Surg* 1996; **224**: 66-71
45 **Rice TW**, Falk GW, Achkar E, Petras RE. Surgical management of high-grade dysplasia in Barrett's esophagus. *Am J Gastroenterol* 1993; **88**: 1832-1836
46 **Conio M**, Cameron AJ, Chak A, Blanchi S, Filiberti R. Endoscopic treatment of high-grade dysplasia and early cancer in Barrett's oesophagus. *Lancet Oncol* 2005; **6**: 311-321
47 **Ell C**, May A, Gossner L, Pech O, Günter E, Mayer G, Henrich R, Vieth M, Müller H, Seitz G, Stolte M. Endoscopic mucosal resection of early cancer and high-grade dysplasia in Barrett's esophagus. *Gastroenterology* 2000; **118**: 670-677
48 **May A**, Gossner L, Pech O, Fritz A, Günter E, Mayer G, Müller H, Seitz G, Vieth M, Stolte M, Ell C. Local endoscopic therapy for intraepithelial high-grade neoplasia and early adenocarcinoma in Barrett's oesophagus: acute-phase and intermediate results of a new treatment approach. *Eur J Gastroenterol Hepatol* 2002; **14**: 1085-1091
49 **Hull MJ**, Mino-Kenudson M, Nishioka NS, Ban S, Sepehr A, Puricelli W, Nakatsuka L, Ota S, Shimizu M, Brugge WR, Lauwers GY. Endoscopic mucosal resection: an improved diagnostic procedure for early gastroesophageal epithelial neoplasms. *Am J Surg Pathol* 2006; **30**: 114-118
50 **Conio M**, Repici A, Cestari R, Blanchi S, Lapertosa G, Missale G, Della Casa D, Villanacci V, Calandri PG, Filiberti R. Endoscopic mucosal resection for high-grade dysplasia and intramucosal carcinoma in Barrett's esophagus: an Italian experience. *World J Gastroenterol* 2005; **11**: 6650-6655

51 **Conio M**, Ponchon T, Blanchi S, Filiberti R. Endoscopic mucosal resection. *Am J Gastroenterol* 2006; **101**: 653-663

52 **Katada C**, Muto M, Manabe T, Boku N, Ohtsu A, Yoshida S. Esophageal stenosis after endoscopic mucosal resection of superficial esophageal lesions. *Gastrointest Endosc* 2003; **57**: 165-169

53 **Seewald S**, Akaraviputh T, Seitz U, Brand B, Groth S, Mendoza G, He X, Thonke F, Stolte M, Schroeder S, Soehendra N. Circumferential EMR and complete removal of Barrett's epithelium: a new approach to management of Barrett's esophagus containing high-grade intraepithelial neoplasia and intramucosal carcinoma. *Gastrointest Endosc* 2003; **57**: 854-859

54 **Pacifico RJ**, Wang KK, Wongkeesong LM, Buttar NS, Lutzke LS. Combined endoscopic mucosal resection and photodynamic therapy versus esophagectomy for management of early adenocarcinoma in Barrett's esophagus. *Clin Gastroenterol Hepatol* 2003; **1**: 252-257

55 **Cameron AJ**, Carpenter HA. Barrett's esophagus, high-grade dysplasia, and early adenocarcinoma: a pathological study. *Am J Gastroenterol* 1997; **92**: 586-591

56 **Ahmad NA**, Kochman ML, Long WB, Furth EE, Ginsberg GG. Efficacy, safety, and clinical outcomes of endoscopic mucosal resection: a study of 101 cases. *Gastrointest Endosc* 2002; **55**: 390-396

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RAPID COMMUNICATION

Assessment of oxidative stress in chronic pancreatitis patients

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Abstract

AIM: To assess the levels of antioxidant capacity and oxidative damage in blood of chronic pancreatitis (CP) patients in comparison with those in healthy control subjects, by using several different analytical techniques.

METHODS: Thirty-five CP patients and 35 healthy control subjects were investigated prospectively with respect to plasma levels of thiols, ferric reducing ability of plasma (FRAP, i.e. antioxidant capacity), levels of protein carbonyls and thiobarbituric acid reactive substances (TBARS). Additionally, we evaluated the production of reactive oxygen species (ROS) in whole blood.

RESULTS: The antioxidative thiols including cysteine, cysteinylglycine and glutathione were significantly lower in CP patients. In addition, the non-enzymatic antioxidant capacity was significantly lower in CP patients, which correlated with the amount of oxidative protein (protein carbonyls) and the extent of lipid damage (TBARS), both were significantly higher in CP patients. The ROS production in whole blood after stimulation with phorbol 12-myritate 13-acetaat, demonstrated a strong tendency to produce more ROS in CP patients.

CONCLUSION: Oxidative stress may contribute to the pathogenesis of chronic pancreatitis by decreasing antioxidant capacity and increasing oxidative damage in CP patients may be a rationale for intervention with antioxidant therapy.

Key words: Chronic pancreatitis; Oxidative stress; Thiols; Ferric reducing ability of plasma; Protein carbonyls; Thiobarbituric acid reactive substances; Reactive oxygen species

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive irreversible inflammatory disease that eventually leads to an impaired exocrine and/or endocrine function of the pancreas[1-4]. Although most cases have been attributed to alcohol abuse, the underlying causes of CP appear to be multi-faceted, including environmental as well as genetic factors. Chronic pancreatitis shares risk factors with pancreatic cancer such as smoking and alcohol abuse, but itself is also a risk factor for pancreatic adenocarcinoma[5]. A genetic predisposition to pancreatitis is supported by the identification of sequence alterations in the genes encoding cationic trypsinogen (PRSS1), the cystic fibrosis transmembrane conductance regulator (CFTR), and the serine protease inhibitor, Kazal type 1 (SPINK1) in patients with hereditary or idiopathic chronic pancreatitis[1,6-8]. Additionally, an increased frequency of SPINK1 mutations been reported in patients with alcohol-related chronic pancreatitis[3,9]. So far we have not completely understood the pathogenesis of CP[10]. Different hypotheses have been proposed, including the contribution of oxidative stress of endogenous origin or chemical stress by environmental or lifestyle-related xenobiotics[11-15]. There is growing recognition that an imbalance between reactive oxygen species (ROS) producing and ROS scavenging processes leads to the damage of pancreatic acinar cells, initiating auto-digestion of the entire pancreas. This insight is suggested by data from experimental and clinical studies[16-19]. Oxidative stress may be important in the pathogenesis of ethanol-induced pancreatic injury, although radiation, exposure to cigarette smoke, medication or trauma may stimulate the generation of free radicals, which subsequently may result in damage of lipids, proteins or nucleic acids. Activation of (enzymatic) antioxidative defence has been described in pancreatic disease independent of its origin[20]. Glutathione and cysteine are important mediators in the defence against oxidative stress and both molecules play a key role in the maintenance of cellular thiol redox status. Therefore, in the present study the concentrations of glutathione, cysteine and other thiols were measured in blood plasma of patients with CP

and healthy control subjects. In addition, we also measured the non-enzymatic antioxidant capacity by applying the ferric reducing ability of plasma (FRAP) assay in patients with CP and healthy controls. Further assessment of the level of oxidative stress was performed by measuring the concentrations of protein carbonyls in plasma in order to determine the amount of oxidative protein damage. As an indicator of lipid peroxidation we established the concentrations of thiobarbituric acid-reactive substances (TBARS). Finally, we investigated the generation of ROS by chemiluminescence in whole blood of both patients and controls.

MATERIALS AND METHODS

Subjects

The study was approved by the local medical ethical review committee and all subjects gave their written informed consent. This study was conducted at the Department of Gastroenterology of the University Medical Centre Nijmegen, the Netherlands and all subjects studied were Caucasians of Dutch extraction. A total of 35 consecutive CP patients were recruited between January 2004 and June 2004 at the out-patient clinic of the department. In 29 patients an alcohol-related etiology was indicated (ACP), the remaining 6 CP patients had a family history of CP (HCP). The clinical diagnosis of CP was based on one or more of the following criteria: presence of typical complaints (recurrent upper abdominal pain, radiating to the back, relieved by leaning forward or sitting upright and increased after eating), suggestive radiological findings such as pancreatic calcifications or pseudocysts, and pathological findings (pancreatic ductal irregularities and dilatations) revealed by endoscopic retrograde pancreaticography or magnetic resonance imaging of the pancreas before and after stimulation with secretin. ACP was diagnosed in patients who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than two years before they were diagnosed, during their treatment they all gave up drinking alcohol. HCP was diagnosed based on the presence of two first-degree relatives or three or more second-degree relatives in two or more generations, suffering from recurrent acute pancreatitis or chronic pancreatitis for which there was no precipitating factor. For comparison, we collected a control group consisting of 35 healthy subjects. We recruited our healthy controls by advertisement in a local paper and did not apply any monetary incentive for the controls to participate.

Analysis of thiols

Samples of blood were taken by venapuncture into EDTA tubes. Whole blood was centrifuged at 1500 × *g* for 10 min within 1 h after collection and plasma was stored at -30℃ until analysis. Concentrations of the thiols including cysteine, homocysteine, cysteinylglycine and glutathione (the sum of reduced-, oxidised- and protein-bound thiols) in plasma were quantified using high performance liquid chromatography (HPLC) with fluorescent detection, essentially as described by Fortin *et al*[21] and modified by Raijmakers *et al*[22]. Thiol levels were calculated using four-point calibration curves for each thiol, which were run in parallel with the samples, and values were expressed in μmol/L.

Analysis of FRAP

The antioxidant capacity in blood plasma was measured using the ferric reducing ability of plasma (FRAP) assay, according to the method of Benzie and Strain[23]. The reduction of ferric to ferrous ion at low pH formed a coloured ferrous-tripyridyltriazine complex. Absorbance changes were linear over a wide concentration range with antioxidant mixtures, including plasma. FRAP values were obtained using a seven-point calibration curve of known amounts of Fe^{2+} and expressed in mmol Fe^{2+}/L.

Analysis of protein carbonyls

The amount of oxidative protein damage, as a marker for oxidative stress, was determined using an enzyme linked immunosorbent assay (ELISA) for estimation of protein carbonyls in body fluids, as essentially described by Buss *et al*[24] and adapted by Zusterzeel *et al*[25]. Samples were incubated with dinitrophenylhydrazine and then adsorbed to wells of an ELISA plate before probing with a commercial antibody raised against protein-conjugated dinitrophenylhydrazine. The binding of biotin-conjugated primary antibody was then quantified after incubation with streptavidin-biotinylated horseradish peroxidase and staining with o-phenylenediamine. Calibration took place using oxidised and fully reduced albumin, and carbonyl levels were expressed in μmol/g protein.

Analysis of TBARS

Thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde (MDA) in plasma were evaluated by recording the fluorescence spectrum between 500 and 600 nm on a Shimadzu RFF-5000 spectrofluorometer, of the thiobarbituric acid-malonaldehyde complex, as described by Conti *et al*[26]. Levels of TBARS were expressed in μmol MDA/L.

Analysis of ROS

ROS production in whole blood was evaluated using luminol- enhanced chemiluminescence, as measured in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Belgium). Briefly, the signal-amplifying molecule luminol reacts with oxygen species (mainly superoxide anion) generated by neutrophils in whole blood, to produce an excited state intermediate that emits light as it returns to its ground state. ROS production was determined in the absence of a cellular stimulator, as well as in the presence of either a receptor-dependent (serum-treated zymosan, STZ) or a receptor-independent stimulus (phorbol 12-myritate 13-acetaat, PMA). Freshly obtained heparinized blood was 1:100 diluted in HBSS containing 1 mmol/L calcium. Two hundred μL of this diluted blood was added to each well of a 96-well plate. In addition, reaction mixtures contained 0.45 g/L bovine serum albumin (BSA), 0.83 mmol/L luminol and either 1 g/L STZ, 0.4 mg/L PMA or no stimulating agents. As an internal positive control for the luminescence process, samples of 1 g/L ammonium persulphate (APS) in phosphate-buffered

solution (PBS) were run simultaneously. Chemiluminescence was monitored every 60 s for 1 h. EDTA blood, taken together with the heparinized blood samples, was tested for leukocyte counts and differentiation, in order to adjust the chemiluminescence produced during one hour ('area under the curve') in relative light units (RLU) per cell for neutrophil counts. All measurements were performed in quadruplicate and corrected for background values (absence of a stimulus). Opsonized zymosan particles were prepared by incubation of STZ with pooled human serum for 30 min at 37℃, as previously described[27]. These particles were then washed twice in PBS and finally suspended at 12.5 g/L in PBS.

Statistical analysis

Data were analysed using SPSS version 12.0. Differences in the baseline characteristics of patients and controls were estimated with Fisher's exact test and Student's *t*-test. The Mann-Witney U-test was used to estimate differences in biochemical parameters between the patient and control population in a non-parametrical manner. Differences were considered significant if $P < 0.05$. Finally, we examined the correlation between the non-enzymatic antioxidant capacity with the amount of oxidative protein and lipid damage in CP patients, using Spearman rank correlation test.

RESULTS

The characteristics of patients with CP and healthy controls are denoted in Table 1. The mean age of the CP patients was 51 years (range 25 to 74 years) and was not significantly different from that of the healthy controls (45 years; range 27 to 68 years). There was no significant difference in the distribution of gender between CP patients and healthy control subjects. Smoking habits between CP patients and healthy controls were not different; 66% of the patients and 63% of the control subjects smoked or stopped smoking within the last 5 years.

The oxidative stress was measured in CP patients and healthy controls. The plasma concentrations of cysteine (Cys), homocysteine (Hcys), cysteinylglycine (CGS) and glutathione (GSH), the plasma antioxidant capacity (FRAP) as well as the plasma levels of protein carbonyls and TBARS and chemoluminescense in whole blood are depicted in Table 2.

The plasma concentrations of antioxidative thiols including cysteine, cysteinylglycine and glutathione were significantly lower in the CP patients than in the controls ($P = 0.021$, $P = 0.003$ and $P = 0.048$, respectively). The plasma levels of homocysteine were similar in both groups. The antioxidant capacity as measured by the FRAP assay was also significantly lower in the CP patients than in the healthy control subjects ($P < 0.001$). The levels of both carbonyls and TBARS were significantly higher in the CP patients than in the healthy controls ($P < 0.001$). The chemiluminescence of diluted whole blood of CP patients and controls was not different, although there was a strong trend towards an increased ROS production after stimulation with PMA ($P = 0.058$). As expected, spontaneous generation of ROS in the absence of a stimulus was less than 10% of the amount of ROS measured in response to the stimuli of PMA and STZ. The leukocyte counts and differentiation within the ranges were considered normal at our hospital. The values obtained with either assay were not different in patients with ACP and HCP.

In addition, the correlation between the non-enzymatic antioxidant capacity with the amount of oxidative protein and lipid damage in CP patients was examined and a negative correlation was found between the non-enzymatic antioxidant capacity and the amount of oxidative protein damage ($r_s = -0.44$, $P = 0.013$), as well as between the non-enzymatic antioxidant capacity and the amount of lipid damage ($r_s = -0.39$, $P = 0.004$). Finally, we found a positive correlation between oxidative protein and lipid damage ($r_s = 0.67$, $P = 0.001$).

Table 1 Main characteristics of patients with chronic pancreatitis and healthy controls

Characteristic	CP patients	Controls
n	35	35
Gender		
Male/Female	17/18	18/17
Mean age (yr) (range)	51(25-74)	45 (27- 68)
Smoking		
Yes/No	23/12	22/13

Table 2 Thiol plasma concentrations, FRAP antioxidant capacity, protein carbonyl plasma levels, TBARS plasma levels and ROS production in whole blood of patients with CP and controls mean (range)

Measure for oxidative stress	CP patients	Controls
Cys[1] (μmol/L)	225 (124-314)[a]	249 (212-328)
Hcys[2] (μmol/L)	13.6 (5.0-38.2)	12.7 (0.2-27.8)
CGS[3] (μmol/L)	34.8 (23.5-124)[a]	39.3 (25.2-56.7)
GSH[4] (μmol/L)	7.5 (2.4-18.5)[a]	8.9 (3.5-16.1)
FRAP[5] (mmol Fe^{2+}/L)	0.75 (0.31-1.73)[a]	0.99 (0.69-1.57)
Carbonyls (nmol/mg protein)	0.32 (0.02-1.47)[a]	0.04 (0.01-0.07)
TBARS[6] (μmol/L)	4.98 (0.23-27.79)[a]	0.35 (0.04-0.68)
ROS[7] production (RLU/10^4 cells)		
PMA[8]-stimulated	40 (1-83)	33 (1-87)
STZ[9]-stimulated	124 (11-266)	111 (9-2130)

[1]Cysteine; [2]Homocysteine; [3]Cysteinylglycine; [4]Glutathione; [5]Ferric reducing ability of plasma; [6]Thiobarbituric acid-reactive substances; [7]Reactive oxygen species; [8]Phorbol 12-myristate 13-acetate; [9]Serum treated zymosan. [a]$P < 0.05$ *vs* control.

DISCUSSION

Alcohol abuse is regarded as a major risk factor for the development of CP. However, the exact mechanism behind the effect of alcohol remains unknown. Some evidence obtained by animal studies, suggests that metabolism of ethanol catalysed by cytochrome P4502E1 (CYP2E1) may contribute to oxidative stress in the pancreas during chronic alcohol consumption[19]. Trauma, exposure to radiation, cigarette smoke, medication or other toxins generating free

radicals also may increase the amount of oxidants. In the present study, we assessed the antioxidant capacity and levels of oxidative damage in CP patients as compared with healthy controls by means of several analytical techniques that are known to measure various components that together constitute oxidative stress. The major observations were that plasma concentrations of some thiols, having antioxidant properties, were significantly lower in CP patients. Likewise the non-enzymatic antioxidant capacity as measured by the FRAP assay, was significantly lower in CP patients than in healthy control subjects. This inferior antioxidant capacity in CP patients parallels significantly increased amounts of oxidative protein and lipid damage, whereas the generation of ROS in whole blood did not show a statistically significant difference between CP patients and healthy control subjects, with a similar age and gender distribution and smoking habits. Our results clearly indicate that oxidative stress is present in patients with CP and that this eventually might contribute to the initiation and maintenance of inflammation in CP patients, as has been previously suggested[17,19,28-31]. Thiols such as cysteine and glutathione play an essential role as antioxidants, and are involved in protein synthesis, redox sensitive transduction signalling, cell growth and proliferation, xenobiotic metabolism and immune regulation[32]. Glutathione is conjugated to many xenobiotics and essential for the optimal functioning of numerous enzymes and hence crucial for cell viability. Decreased levels of glutathione in plasma have been reported, but paradoxically also increased glutathione levels may be found as a result of outshoot after enhanced synthesis due to oxidative stress or conjugation of toxic compounds, as has been shown in different disorders[33-35]. We found significantly lower plasma concentrations of cysteine, cysteinylglycine and glutathione in CP patients as compared to healthy controls, whereas the control values measured here can be considered normal for the Dutch population[36]. We found no elevated concentrations of homocysteine in CP patients, however homocysteine does not always act as an antioxidant, moreover elevated plasma concentrations of homocysteine are positively associated with an increased risk of cerebral, coronary or peripheral vascular diseases[36]. In parallel with the lower plasma concentrations of the antioxidative thiols, the FRAP assay also demonstrated a significantly lower antioxidant capacity in CP patients. Since the FRAP assay does not measure sulfhydryl (SH)-containing antioxidants such as the thiols glutathione and cysteine, this indicates that other non-thiol related antioxidants are decreased in CP patients also. In patients with acute pancreatitis very low ascorbate concentrations in plasma have been described before [37,38].

Protein carbonyl derivates are generated by direct oxidative attack of proteins or by indirect lipid peroxidation products and therefore represent a good biomarker for general oxidative stress[39,40]. The lower FRAP levels in CP patients are accompanied with high protein carbonyl concentrations, indicating that increased oxidative damage occurs as a result of the lower protective capacity as measured by FRAP. Former studies have demonstrated elevated plasma protein carbonyls in experimental animal models[41,42] and humans with acute pancreatitis[43]. Unless properly scavenged, ROS may lead to lipid peroxidation, which represents an important manifestation of oxidative stress. Lipid peroxidation is initiated when free radicals interact with polyunsaturated fatty acids. For instance, in cell membranes this may result in a chain reaction forming lipid hydroperoxides. Analysis of TBARS in plasma is a widely used method for the estimation of lipid peroxidation. In accordance with the elevated levels of protein carbonyls and lower antioxidant capacity, we found significantly increased plasma concentrations of TBARS in CP patients. The production of ROS, as measured in whole blood by chemiluminescence assay, was not significantly higher in CP patients than in healthy controls, although there was a strong trend to generate higher amounts of ROS after stimulation with PMA. We used luminol-enhanced chemiluminescence assay in 100-fold diluted whole blood to study ROS generation of peripheral blood cells in a non-disturbed system. As expected, ROS generation in the absence of a stimulus in CP patients was low, and not significantly different from the values in the healthy control group. Chemiluminescence measurements did not demonstrate a significantly increased ROS generation in CP patients, while the other analytical techniques applied here showed increased oxidative stress and damage in CP patients as compared to healthy control subjects, demonstrating that the oxidative damage in CP patients is caused by other reactive (oxygen) species rather than by leucocytes. Since most of the CP patients included in this study were alcoholics, the cause of oxidative damage might be mainly of exogenous origin. However, a contribution of oxidative stress of endogenous origin might also be possible, since we detected a strong tendency to produce ROS in CP patients after stimulation with PMA. This PMA-induced respiratory burst is receptor-independent and not absolutely dependent on priming[44], whereas priming does moderately influence the STZ-induced respiratory burst[45]. We measured the ROS production in whole blood and it is known that during isolation of neutrophils, these cells often become primed[46]. The clinical importance of oxidative stress in human pancreatic disease was first suggested by Braganza *et al*[47], and subsequently supported by data from other groups, showing increased levels of lipid hydroperoxides in pancreatic juice[14] and increased spontaneous production of ROS by neutrophils[27,48]. The assessment of oxidative stress in CP patients corroborates the hypothesis that oxidative stress leads to damage of pancreatic acinar cells, initiating auto-digestion of the entire pancreas as has been shown in the present study.

In summary, significantly higher levels of products of oxidative damage (protein carbonyls and TBARS), correlating with decreased levels of cysteine, cysteinylglycine, glutathione and non-enzymatic antioxidant capacity (FRAP) can be found in CP patients. Oxidative stress, defined as the imbalance between prooxidant and antioxidant capacity, is higher in CP patients, which may justify further studies on intervention with antioxidant therapies for this serious disease.

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REFERENCES

1 **Witt H**, Luck W, Hennies HC, Classen M, Kage A, Lass U, Landt O, Becker M. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat Genet* 2000; **25**: 213-216
2 **Etemad B**, Whitcomb DC. Chronic pancreatitis: diagnosis, classification, and new genetic developments. *Gastroenterology* 2001; **120**: 682-707
3 **Drenth JP**, te Morsche R, Jansen JB. Mutations in serine protease inhibitor Kazal type 1 are strongly associated with chronic pancreatitis. *Gut* 2002; **50**: 687-692
4 **Jansen JB**, te Morsche R, van Goor H, Drenth JP. Genetic basis of chronic pancreatitis. *Scand J Gastroenterol Suppl* 2002; (**236**): 91-94
5 **Lowenfels AB**, Maisonneuve P, Lankisch PG. Chronic pancreatitis and other risk factors for pancreatic cancer. *Gastroenterol Clin North Am* 1999; **28**: 673-85, x
6 **Whitcomb DC**, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK Jr, Amann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, Ehrlich GD. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* 1996; **14**: 141-145
7 **Sharer N**, Schwarz M, Malone G, Howarth A, Painter J, Super M, Braganza J. Mutations of the cystic fibrosis gene in patients with chronic pancreatitis. *N Engl J Med* 1998; **339**: 645-652
8 **Cohn JA**, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell PS. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. *N Engl J Med* 1998; **339**: 653-658
9 **Witt H**, Luck W, Becker M, Böhmig M, Kage A, Truninger K, Ammann RW, O'Reilly D, Kingsnorth A, Schulz HU, Halangk W, Kielstein V, Knoefel WT, Teich N, Keim V. Mutation in the SPINK1 trypsin inhibitor gene, alcohol use, and chronic pancreatitis. *JAMA* 2001; **285**: 2716-2717
10 **Steer ML**, Waxman I, Freedman S. Chronic pancreatitis. *N Engl J Med* 1995; **332**: 1482-1490
11 **Bulger EM**, Helton WS. Nutrient antioxidants in gastrointestinal diseases. *Gastroenterol Clin North Am* 1998; **27**: 403-419
12 **Schoenberg MH**, Birk D, Beger HG. Oxidative stress in acute and chronic pancreatitis. *Am J Clin Nutr* 1995; **62**: 1306S-1314S
13 **Ulrich AB**, Schmied BM, Matsuzaki H, Lawson TA, Friess H, Andrén-Sandberg A, Büchler MW, Pour PM. Increased expression of glutathione S-transferase-pi in the islets of patients with primary chronic pancreatitis but not secondary chronic pancreatitis. *Pancreas* 2001; **22**: 388-394
14 **Santini SA**, Spada C, Bononi F, Foschia F, Mutignani M, Perri V, Giardina B, Silveri NG, Costamagna G. Liver, pancreas and biliary tract enhanced lipoperoxidation products in pure pancreatic juice: evidence for organ-specific oxidative stress in chronic pancreatitis. *Dig Liver Dis* 2003; **35**: 888-892
15 **Seicean A**, Grigorescu M. The pathogenesis of chronic alcoholic pancreatitis. *Rom J Gastroenterol* 2002; **11**: 19-24
16 **Matsumura N**, Ochi K, Ichimura M, Mizushima T, Harada H, Harada M. Study on free radicals and pancreatic fibrosis--pancreatic fibrosis induced by repeated injections of superoxide dismutase inhibitor. *Pancreas* 2001; **22**: 53-57
17 **Mathew P**, Wyllie R, Van Lente F, Steffen RM, Kay MH. Antioxidants in hereditary pancreatitis. *Am J Gastroenterol* 1996; **91**: 1558-1562
18 **Wallig MA**. Xenobiotic metabolism, oxidant stress and chronic pancreatitis. Focus on glutathione. *Digestion* 1998; **59** Suppl 4: 13-24
19 **Norton ID**, Apte MV, Lux O, Haber PS, Pirola RC, Wilson JS. Chronic ethanol administration causes oxidative stress in the rat pancreas. *J Lab Clin Med* 1998; **131**: 442-446
20 **Simovic MO**, Bonham MJ, Abu-Zidan FM, Windsor JA. Manganese superoxide dismutase: a marker of ischemia-reperfusion injury in acute pancreatitis? *Pancreas* 1997; **15**: 78-82
21 **Fortin LJ**, Genest J Jr. Measurement of homocyst(e)ine in the prediction of arteriosclerosis. *Clin Biochem* 1995; **28**: 155-162
22 **Raijmakers MT**, Zusterzeel PL, Steegers EA, Hectors MP, Demacker PN, Peters WH. Plasma thiol status in preeclampsia. *Obstet Gynecol* 2000; **95**: 180-184
23 **Benzie IF**, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 1996; **239**: 70-76
24 **Buss H**, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. *Free Radic Biol Med* 1997; **23**: 361-366
25 **Zusterzeel PL**, Rütten H, Roelofs HM, Peters WH, Steegers EA. Protein carbonyls in decidua and placenta of pre-eclamptic women as markers for oxidative stress. *Placenta* 2001; **22**: 213-219
26 **Conti M**, Morand PC, Levillain P, Lemonnier A. Improved fluorometric determination of malonaldehyde. *Clin Chem* 1991; **37**: 1273-1275
27 **Goldstein IM**, Roos D, Kaplan HB, Weissmann G. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J Clin Invest* 1975; **56**: 1155-1163
28 **Tsuji N**, Watanabe N, Okamoto T, Niitsu Y. Specific interaction of pancreatic elastase and leucocytes to produce oxygen radicals and its implication in pancreatitis. *Gut* 1994; **35**: 1659-1664
29 **Szuster-Ciesielska A**, Daniluk J, Kandefer-Szerszeń M. Oxidative stress in blood of patients with alcohol-related pancreatitis. *Pancreas* 2001; **22**: 261-266
30 **Aleynik SI**, Leo MA, Aleynik MK, Lieber CS. Alcohol-induced pancreatic oxidative stress: protection by phospholipid repletion. *Free Radic Biol Med* 1999; **26**: 609-619
31 **Guyan PM**, Uden S, Braganza JM. Heightened free radical activity in pancreatitis. *Free Radic Biol Med* 1990; **8**: 347-354
32 **Sen CK**. Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem Pharmacol* 1998; **55**: 1747-1758
33 **Gut A**, Chaloner C, Schofield D, Sandle LR, Purmasir M, Segal I, Braganza JM. Evidence of toxic metabolite stress in black South Africans with chronic pancreatitis. *Clin Chim Acta* 1995; **236**: 145-153
34 **Plummer JL**, Smith BR, Sies H, Bend JR. Chemical depletion of glutathione in vivo. *Methods Enzymol* 1981; **77**: 50-59
35 **Deneke SM**, Fanburg BL. Regulation of cellular glutathione. *Am J Physiol* 1989; **257**: L163-L173
36 Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA* 2002; **288**: 2015-2022
37 **Scott P**, Bruce C, Schofield D, Shiel N, Braganza JM, McCloy RF. Vitamin C status in patients with acute pancreatitis. *Br J Surg* 1993; **80**: 750-754
38 **Bonham MJ**, Abu-Zidan FM, Simovic MO, Sluis KB, Wilkinson A, Winterbourn CC, Windsor JA. Early ascorbic acid depletion is related to the severity of acute pancreatitis. *Br J Surg* 1999; **86**: 1296-1301
39 **Berlett BS**, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 1997; **272**: 20313-20316
40 **Stadtman ER**, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab Rev* 1998; **30**: 225-243
41 **Reinheckel T**, Nedelev B, Prause J, Augustin W, Schulz HU, Lippert H, Halangk W. Occurrence of oxidatively modified proteins: an early event in experimental acute pancreatitis. *Free Radic Biol Med* 1998; **24**: 393-400
42 **Reinheckel T**, Prause J, Nedelev B, Augustin W, Schulz HU, Lippert H, Halangk W. Oxidative stress affects pancreatic proteins during the early pathogenesis of rat caerulein pancreatitis. *Digestion* 1999; **60**: 56-62
43 **Winterbourn CC**, Bonham MJ, Buss H, Abu-Zidan FM, Windsor JA**.** Elevated protein carbonyls as plasma markers of oxidative stress in acute pancreatitis. *Pancreatology* 2003; **3**: 375-382
44 **Koenderman L**, Yazdanbakhsh M, Roos D, Verhoeven AJ. Dual mechanisms in priming of the chemoattractant-induced

respiratory burst in human granulocytes. A Ca2+-dependent and a Ca2+-independent route. *J Immunol* 1989; **142**: 623-628

45 **Tool AT**. Priming and activation of human granulocytes [dissertation]. The Netherlands: University of Amsterdam, 1996

46 **Kuijpers TW**, Tool AT, van der Schoot CE, Ginsel LA, Onderwater JJ, Roos D, Verhoeven AJ. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* 1991; **78**: 1105-1111

47 **Braganza JM**, Scott P, Bilton D, Schofield D, Chaloner C, Shiel N, Hunt LP, Bottiglieri T. Evidence for early oxidative stress in acute pancreatitis. Clues for correction. *Int J Pancreatol* 1995; **17**: 69-81

48 **Szuster-Ciesielska A**, Daniluk J, Kandefer-Szerszeń M. Alcohol-related cirrhosis with pancreatitis. The role of oxidative stress in the progression of the disease. *Arch Immunol Ther Exp (Warsz)* 2001; **49**: 139-146

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RAPID COMMUNICATION

Chronic Epstein-Barr virus-related hepatitis in immunocompetent patients

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Abstract

AIM: To investigate reactivated Epstein-Barr virus (EBV) infection as a cause for chronic hepatitis.

METHODS: Patients with occasionally established elevated serum aminotransferases were studied. HIV, HBV and HCV-infections were excluded as well as any other immunosuppressive factors, metabolic or toxic disorders. EBV viral capsid antigen (VCA) IgG and IgM, EA-R and EA-D IgG and Epstein-Barr nuclear antigen (EBNA) were measured using IFA kits. Immunophenotyping of whole blood was performed by multicolor flow cytometry. $CD8^+$ T cell responses to EBV and PHA were determined according to the intracellular expression of IFN-γ.

RESULTS: The mean alanine aminotransferase (ALT) and gamma glutamyl transpeptidase (GGTP) values exceeded twice the upper normal limit, AST/ALT ratio < 1. Serology tests showed reactivated EBV infection in all patients. Absolute number and percentages of T, B and NK cells were within the reference ranges. Fine subset analysis, in comparison to EBV^+ healthy carriers, revealed a significant decrease of naive T cells ($P < 0.001$), accompanied by increased percentage of $CD45RA^-$ ($P < 0.0001$), and terminally differentiated $CD28^-CD27^-$ $CD8^+$ T cells ($P < 0.01$). Moderately elevated numbers of CD38 molecules on $CD8^+$ T cells ($P < 0.05$) proposed a low viral burden. A significantly increased percentage of $CD8^+$ T cells expressing IFN-γ in response to EBV and PHA stimulation was registered in patients, as compared to controls ($P < 0.05$). Liver biopsy specimens from 5 patients revealed nonspecific features of low-grade hepatitis.

CONCLUSION: Chronic hepatitis might be a manifestation of chronic EBV infection in the lack of detectable immune deficiency; the expansion of $CD28^-$ $CD27^-$ and increase of functional EBV-specific $CD8^+$ T cells being the only surrogate markers of viral activity.

Key words: Chronic hepatitis; Epstein-Barr; Epstein-Barr virus-specific $CD8^+$ T cell

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INTRODUCTION

Epstein-Barr virus (EBV) infects more than 90% of humans by the time of adulthood and persists as a life-long latency, suggesting sophisticated mechanisms for effective immune evasion. Primary EBV infection is usually asymptomatic or results in infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity. Similar to other herpes viruses, EBV persists in the infected immunocompetent organism, without causing apparent disease. $CD8^+$ T cell-mediated responses control the virus both during primary infection and the following carrier state[1,2].

So far, studies on the interaction between the immune system and EBV have focused mostly on congenital or acquired immune deficiency states, which can lead to viral reactivation. Rare cases of fulminant chronic EBV infection have been described and termed severe chronic active EBV infection (SCAEBV or CAEBV)[3,4]. Criteria of CAEBV include severe infection lasting more than 6 mo, histologic evidence of major organ involvement, abnormal EBV antibody titers, and increased quantities of EBV in affected tissues in apparently immunocompetent hosts[5]. However, congenital or acquired defects of cell-mediated immune mechanisms in CAEBV patients are very probable, associated with signalling defects, reduced T-cell or NK cell activity, or reduced perforin levels[6].

While hepatitis is a common feature of primary EBV infection[7,8], the role of EBV in chronic liver disease is less obvious. Bertolini *et al* showed typical histopathological lesions of chronic hepatitis without involvement of other organs after inoculation of normal human bone marrow-derived B cell, carrying an endogenous EBV line *in vivo* in nu/nu mice[9]. Single cases of persistent hepatitis related to EBV infection were reported in immunocompromised persons[10]. Liver injury may be one of the manifestations of CAEBV[11-13]. However, only limited knowledge exists about EBV-related hepatitis in immunocompetent patients. We analyzed the clinical and laboratory findings in 15 patients with chronic hepatitis and no data of immune deficiency. Based on the serological profile of reactivated EBV infection and the absence of other major etiological agents, we propose reactivated EBV infection as a cause for the chronic liver disease. Furthermore, we demonstrate that the only significant changes at the level of peripheral blood lymphocytes were the prevalence of $CD45RA^-$ T cells, combined with expansion of $CD8^+$ T cells lacking CD27 and CD28 expression, and an increased percentage of lytic EBV epitope-specific CTL.

MATERIALS AND METHODS

Patients

Fifteen Caucasian HLA-A2+ patients (11 males, 4 females, mean age 40.6 years (range 19-55) with chronic hepatitis, referred to the Clinic of Gastroenterology, Medical Institute, Ministry of Interior, Sofia between Sep 2004 and Mar 2005, were included in this study. Chronic liver injury was detected during routine prophylactic examination and was defined as an elevation of serum aminotransferases of more than 6 mo duration. All patients were HIV-1, HBV and HCV-negative, had normal serum protein levels, and had no evidence of metabolic, or alcohol-related disorders, or drug toxicity (Table 1). Furthermore, they had no history of prior immune deficiency or of any other recent, recurring or chronic infection, or immunosuppressive factors that might explain the observed pathological changes.

A control group included 15 $HLA\text{-}A2^+$ EBV^+ sex- and age-matched healthy volunteers with normal aminotransferase levels. All samples were obtained after informed consent.

Serological and routine laboratory tests

EBV viral capsid antigen (VCA) IgG and IgM, EA-R and EA-D IgG and Epstein-Barr nuclear antigen (EBNA) were measured, using immunofluorescent assay (IFA FDA Atlanta kits). HIV-1, HBsAg, anti-HBc and anti-HCV were tested with ELISA method. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (gamma-GT, GGTP), creatinine, bilirubin, glucose levels, serum proteins and coagulation were measured according to standard laboratory procedures.

Immunophenotypic studies

Whole blood was obtained in heparinized vacutainer tubes. Lymphocyte subset absolute counts were determined by a lysis/no wash procedure with TruCOUNT tubes and CD3/CD4/CD45/CD8 MultiTest; T cell subsets were evaluated by multicolor immunophenotyping and a standard lysis/wash technique (Table 2). The quantitative expression of CD38 on $CD8^+$ T cells (CD38 ABC) was assessed by the QUANTIBRITE PE fluorescence quantitation kit, according to the manufacturer's instructions and analyzed with QUANTIBRITE software. All reagents were products of BD Biosciences (B-D, San Jose, CA). At least 5000 lymphocytes were collected per sample and analysed using FACSCalibur flow cytometer and CellQuest software (B-D).

Table 1 Exclusion criteria

Alcohol consumption > 40 mL per day
Diabetes or impaired glucose tolerance (including hyperinsulinaemia, OGTT and fasting blood glucose)
Under- or overweight (BMI < 18 or > 26 kg/m^2)
Dyslipidaemia
Hyper- or hypothyroidism
Gluten enteropathy
Autoimmune hepatitis or PBC/PSH
Viral hepatitis B or C
Hereditary hemochromatosis or Iron overload
Wilson disease
Anamnesis for hepatotoxic drugs use (including herbal and over-the-counters in the last 12 mo)
Hereditary muscular disorders

Table 2 T cell subsets analyzed in this study

mAb combination	Subset	Phenotype
CCR7/CD45RA/CD8/CD4	Naïve CD8	$CD8^+CD45RA^+CCR7^+$
	Antigen-primed/memory CD8	$CD8^+CD45RA^-$
	Effector CD8	$CD8^+CD45RA^+CCR7^-$
	Naïve CD4	$CD4^+CD45RA^+CCR7^+$
	Antigen-primed/memory CD4	$CD4^+CD45RA^-$
CD27/CD28/CD8	Early CD8	
	Intermediate CD8	$CD8^+CD27^+CD28^+$
	Terminally differentiated CD8	$CD8^+CD27^+CD28^-$

Intracellular cytokine analysis

The HLA-A2 restricted epitope GLCTLVAML from the EBV lytic cycle protein BMLF1 was synthesized according to the standard Fmoc protocol[14]. EBV peptide (10 mg/L) or PHA (Sigma) were used for overnight stimulation of 250 μL whole blood (at 37℃ and humidified 5% CO_2 atmosphere). Two hours later 12.5 mg/L Brefeldin A (BD) was added. Cells were further washed in cold phosphate buffered saline (PBS), incubated with EDTA for 10 min, washed again and processed according to the protocol for surface and intracellular staining. For intracellular staining, 100 μL of stimulated blood was lysed with 1 mL FACS™ Lysing solution (BD) followed by permeabilization with 0.5 mL 1 × FACS permeabilizing solution (BD) for 10 min. After washing with PBS containing 0.5% BSA, a simultaneous staining for IFN-γ/CD69/CD8/CD4 was performed for 30 min at room temperature in the dark.

Table 3 Characteristics of the patients with chronic hepatitis related to Epstein-Barr virus infection

Pt No.	Age (yr)	Sex	Duration of illness (mo)	AST	ALT	ALP	GGT	Serology EBV (IFA)				
								VCA IgG	VCA IgM	EA-R IgG	EA-D IgG	EBNA
1	40	m	24	52	90	300	200	320	10	80	80	0
2	60	m	6	50	187	280	70	160	0	160	160	40
3	27	m	6	31	107	200	20	640	0	160	160	40
4	40	m	6	30	60	300	40	160	0	160	160	40
5	57	f	24	66	84	280	110	320	0	160	160	40
6	55	f	6	66	85	850	171	160	0	80	80	40
7	28	m	6	40	96	200	60	160	10	80	80	20
8	38	m	12	60	60	150	130	640	0	80	80	20
9	30	m	6	50	125	250	180	640	0	80	80	20
10	25	m	6	20	60	225	90	160	0	80	80	20
11	35	m	6	40	80	136	180	160	0	80	80	40
12	31	m	6	50	80	310	80	320	0	160	160	40
13	43	m	10	60	100	300	100	320	10	160	160	0
14	50	f	6	100	200	130	200	640	0	160	160	40
15	50	f	6	80	120	400	200	320	0	160	160	40

For analysis of intracellular staining at least 25 000 $CD8^{high}$ cells were collected. Detection of at least 0.05% IFN-$\gamma^{+}CD69^{+}CD8^{high}$ cells, after subtraction of non-stimulated control values was considered as significant response.

Liver biopsy and imaging

All patients underwent conventional abdominal ultrasonography. Liver biopsy specimens were obtained from 5 patients.

Statistical analysis

Man-Witney non-parametric test was used to evaluate differences between patient and control groups. *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinical findings

The clinical characteristics of each patient are summarized in Table 3. The estimated mean disease duration before the prophylactic examination was 10.8 (min 6, max 36) mo. At the time of diagnosis of the chronic liver disease none of the 15 patients had an infectious mononucleosis-like illness or any other significant complaints except for one, who reported periods of sub-febrile temperature. None of the patients reported previous opportunistic infections or other significant chronic diseases, or congenital immunodeficiency, or immunosuppressive treatment. The average score of Karnofski performance status was 95%[15].

Laboratory tests showed normal blood counts. Mean values (95% CI) are as follows: white blood cell count, 6.61×10^9/L (5.945 to 7.281); red blood cells 5.08×10^6/L (4.7893 to 5.3707); platelets, 243×10^9/L (207.31 to 277.89). The mean range of serum ALT at the time of diagnosis was 103 UI/mL (UNL = 40) (SD 40.9, SE 10.57), AST/ALT ratio < 1, gamma-GT 122 UI/mL (UNL = 48) (SD 62.41, SE 16.12). Normal serum protein and albumin levels were established in all patients, and no hyperbilirubinaemia or coagulation disturbances were found. Fasting glucose levels, as well as the glucose load test were within the reference ranges. A serum insulin level in 12 patients was below upper limit, while 3 patients were not tested. The abdominal ultrasonography series revealed normal liver and spleen image.

EBV-serology at the time of diagnosis

The EBV-specific antibody titers of each patient are shown in Table 2. Serology tests indicated a reactivated EBV infection in all 15 patients. The average EBV VCA IgG titer was 320 UA/mL (160-640), EBNA average titer was 20 UA/mL (10-40), and EA-R and EA-D were elevated (80-160). EBV VCA IgM was not detected in any patient. All controls were EBV VCA IgM-negative.

Lymphocyte subset analysis

The percentage and absolute counts of total lymphocytes, T, B and NK cells, $CD4^+$ T cells as well as the CD4/CD8 ratio were within the established reference ranges for healthy age-matched controls. Using the mAb combinations CD45RA/CCR7/CD8/CD4 and CD27/CD8/CD28, naïve, central and effector memory, as well as terminally differentiated effector T cells (CTL) were defined (Table 2). The fine subset analysis of T cells revealed a significant decrease of naïve ($CD45RA^+CCR7^+$) $CD4^+$ ($P < 0.05$) and $CD8^+$ ($P < 0.01$) T cells accompanied by an increased percentage of memory ($CD45RA^-$) $CD4^+$ ($P < 0.01$) and $CD8^+$ T cells ($P < 0.001$), as well as of the terminally differentiated ($CD28^-CD27^-$) CTL subset ($P < 0.01$) (Figure 1A-E). A moderately increased number of CD38 molecules on $CD8^+$ T cells was established (mean, 2150; min, 1090; max, 4413) as compared to controls (mean, 1522; min, 510; max, 2210; $P < 0.05$), most probably corresponding to a low level viral replication.

Circulating $CD8^+$ T cells, expressing IFN-γ in response to the lytic BMLF1 epitope were registered in all patients, and their percentage was significantly increased (15/15; mean, 0.39%), as compared to EBV-seropositive donors

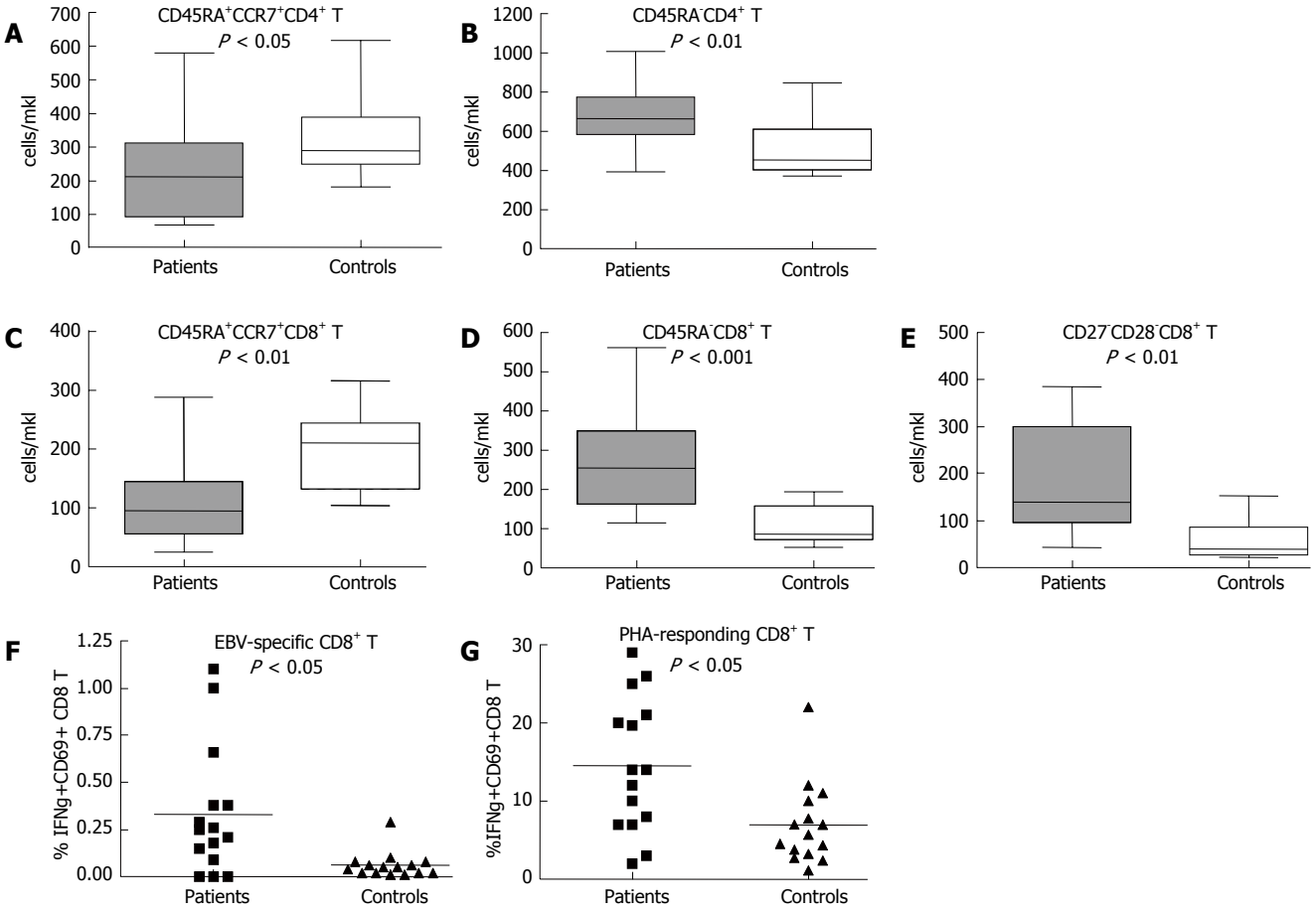

Figure 1 Significant differences detected between patients and control group by immunophenotyping. Patients were characterized by a decreased absolute number of naive CCCR7$^+$CD45RA$^+$CD4$^+$ (**A**) and CD8$^+$ (**C**) T cells, increased absolute number of memory CD45RA$^-$CD4$^+$ (**B**) and CD8$^+$ (**D**)T cells, increased absolute number of effector CD28$^-$CD27$^-$CD8$^+$ T cells (**E**), as well as by increased percentage of IFNγ$^+$CD69$^+$CD8$^+$ T cells after stimulation with EBV peptide GLCTMVL (**F**) and with PHA (**G**). Box-and-whiskers indicate the 25-75 percentile and min-max values for each group. Mean values are designated on all plots by a horizontal line. Man-Witney. *P* values are indicated for each comparison.

(6/15; mean, 0.06%; $P < 0.05$). The percentage of PHA-responding CD8$^+$ T cells was also significantly increased in the patients group in comparison to controls (14.5% *vs* 7.8%, $P < 0.05$) (Figure 1F and G).

Histopathology

Liver biopsy specimens revealed non-specific histopathologic features of low-grade hepatitis. On routine HE slides mild portal and periportal mononuclear infiltrates were seen, composed of small lymphocytes lacking significant cytologic atypia. According to the METAVIR system all patients were staged as A2. No fibrosis was found in any patient except in one which was graded F1 (Figure 2).

DISCUSSION

EBV infects the majority of the human population and persists in the host organism for life. CD8$^+$ T lymphocytes specific for lytic and latent EBV antigens control the virus both during primary infection and the long-term carrier state and almost no symptoms indicate these events[2,16]. Therefore, the development of cellular immune deficiency, related to HIV-infection, transplantation, prolonged immunosuppressive therapy, or even psychological/physical stress is often combined with complications due to EBV reactivation[17]. A broad array of clinical entities has, therefore, been related to EBV infection in immunocompromised host, including persistent and necrotizing hepatitis[18,19]. CAEBV, characterized by a specific pattern of serological and clinical findings, may also induce liver injury[11,12].

We reported the clinical and laboratory features of 15 Caucasian non-related patients with chronic hepatitis in the settings of reactivated EBV infection that had no apparent immune deficiency and did not meet the classical criteria for CAEBV infection. The serological evidence for EBV reactivation was the presence of EBV-NA and EA-R antibodies, combined with elevated but not extreme titers of IgG anti-VCA[20]. None of the patients had significant clinical manifestation of the infection, except for one. Although we did not determine plasma and tissue viral load, the moderately elevated CD38 expression on CD8$^+$ T cells suggested rather a low-level of viral replication[21]. In fact, reactivated EBV infection cases have been reported with mild to moderate clinical presentation, and without extreme EBV-specific Ab titers or viral load[3]. Our data further suggest that the reactivation of EBV infection in the settings of a functional immune system may be more frequent than previously assessed.

Since the latency of EBV infection depends almost entirely on efficient cellular immune responses, phenotypic

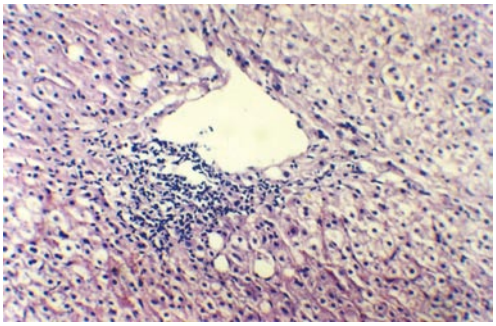

Figure 2 Liver biopsy, showing lymphocyte infiltrate within the lobule and some hepatocyte injury, with mild steatosis displayed in some cells.

and functional changes in the lymphocyte compartment might be expected in case of reactivation. CAEBV is usually characterized by oligoclonal expansions of either T or NK cells[22,23]. Although we detected no significant changes in the main circulating lymphocyte populations, the fine subset analysis revealed a significant prevalence of antigen-primed ($CD45RA^-$) over naïve ($CD45RA^+CCR7^+$) $CD4^+$ and $CD8^+$ T cells, combined with increased percentage of terminally differentiated ($CD28^-CD27^-$) $CD8^+$ T cells. In general, the activation and differentiation state of the circulating T cell pool reflects any major antigen-driven process in the organism. T-cell activation induces a shift from CD45RA to CD45RO expression, while long-lasting chronic viral infections like HIV-1 or CMV are characterized with prevalence of terminally differentiated non-proliferating effectors in the $CD8^+$ T cell compartment[24,25], similar to the natural age-related senescence of cellular immunity[26]. Moreover, particular viruses may be associated with specific phenotypic changes in latency, as the CMV-driven expansion of $CD45RA^+CD27^-CD8^+$ T cells[27]. Logically, such a phenotypic shift must involve both virus-specific and non-specific by-stander activation and differentiation.

The EBV-specific memory pool in asymptomatic carriers is reported to be heterogeneous: $CD8^+$ T cells specific for latent epitopes being mostly $CD45RA^-CD28^+$, while those, specific for lytic epitopes, $CD45RA^+$/$CD45RA^-CD28^-CD27^+$[28]. CD27 is a co-stimulatory molecule promoting the survival of activated $CD8^+$ T cells in the absence of CD28[29], with a limited reexpression capacity. CD27 probably enhances EBV-specific responses, as most of the EBV-specific cytotoxic activity was reported in the $CD27^+$ subpopulation[30]. In our patients group we established a significant expansion of $CD8^+$ T cells lacking both CD28 and CD27. We may speculate that in case of long lasting or repeated low-level EBV reactivation, the $CD27^+$ pool would be gradually replaced by $CD27^-$ $CD8^+$ T cells, less efficient in controlling the persistent EBV infection.

EBV-specific cells detected by tetramer staining in latency account for 1%-3% of the circulating $CD8^+$ T cell pool[31]. Again by tetramer staining, a similar detection rate of lytic epitope-specific $CD8^+$ T cells has been reported in CAEBV and asymptomatic EBV carriers[16]. In our series, patients with EBV-related hepatitis had a significantly higher lytic epitope-specific CTL response, as compared to controls, combined with a higher response to non-specific stimulation, as estimated by IFN-γ expression. These results indicate that the identification of functional EBV-specific $CD8^+$ T cells may better differentiate between asymptomatic carriers and clinically unapparent but active infection.

Several viruses have been implicated in the development of chronic liver disease, either as single agents or *via* cross-activation of T cells. A close association between liver lesions and a previous EBV infection has been demonstrated, as well as the presence of EBV-positive cells in affected livers in the settings of continuous low level EBV replication[32,33]. Sugawara *et al*[34-36] linked EBV to HCV-related hepatocellular cancerogenesis, supporting the EBNA-1 promoted HCV replication hypothesis. EBV was suggested to be involved in the hepatocellular carcinogenesis, although this role is still controversial[37]. Our patients had serological evidence for EBV reactivation and, on the other hand, immunophenotypic evidence for persisting low-level cellular immunity activation and chronic liver inflammation. As the mechanism of EBV-related liver damage remains unclear, indirect, cytokine-induced hepatocellular injury is being discussed[38,39]. Despite the lack of typical mononucleosis characteristics, liver lymphocyte infiltrates in our cases might be involved in the EBV-provoked immune response.

Although based mostly on exclusion criteria, our data suggest that chronic liver disease could be a manifestation of chronic EBV infection, with persistent low-level virus replication or in case of frequent reactivations. It remains to elucidate the underlying mechanisms of inefficient viral control in these immunocompetent patients and whether such patients may further revert to a fulminant course of the infection.

In conclusion, a reactivated EBV infection might be the possible reason for a chronic elevation of serum aminotransferases without a detectable immune deficiency. The prevalence of memory ($CD45RA^-$) and terminally differentiated ($CD28^-CD27^-$) $CD8^+$ T cells together with the increased frequency of functionally active EBV-specific $CD8^+$ T cells in the circulating $CD8^+$ T cell compartment might be the only surrogate markers of reactivated viral infection. The identification and follow-up of such patients may help the prevention of classic CAEBV or other severe manifestations of chronic EBV infection.

REFERENCES

1 **Rickinson AB**, Kieff E. Epsterin-Barr virus. In: Knipe DM, Howley PM, eds. Field's Virology, 4th ed, Vol.2. Philadelphia: Lippincott Williams & Wilkins, 2001: 2575-2627

2 **Cohen JI**. Epstein-Barr virus infection. *N Engl J Med* 2000; **343**: 481-492

3 **Kimura H**, Hoshino Y, Kanegane H, Tsuge I, Okamura T, Kawa K, Morishima T. Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* 2001; **98**: 280-286

4 **Kimura H**, Morishima T, Kanegane H, Ohga S, Hoshino Y, Maeda A, Imai S, Okano M, Morio T, Yokota S, Tsuchiya S, Yachie A, Imashuku S, Kawa K, Wakiguchi H. Prognostic factors for chronic active Epstein-Barr virus infection. *J Infect Dis* 2003; **187**: 527-533

5 **Straus SE**. The chronic mononucleosis syndrome. *J Infect Dis*

1988; **157**: 405-412
6 **Katano H**, Ali MA, Patera AC, Catalfamo M, Jaffe ES, Kimura H, Dale JK, Straus SE, Cohen JI. Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation. *Blood* 2004; **103**: 1244-1252
7 **Kimura H**, Nagasaka T, Hoshino Y, Hayashi N, Tanaka N, Xu JL, Kuzushima K, Morishima T. Severe hepatitis caused by Epstein-Barr virus without infection of hepatocytes. *Hum Pathol* 2001; **32**: 757-762
8 **White NJ**, Juel-Jensen BE. Infectious mononucleosis hepatitis. *Semin Liver Dis* 1984; **4**: 301-306
9 **Bertolini L**, Iacovacci S, Bosman C, Carloni G, Monaco V, Bangrazi C, Serafino A, Gualandi G, Prantera G, Fruscalzo A. Low cell dosage of lymphoblastoid human cell lines EBV(+) is associated to chronic hepatitis in a minority of inoculated Nu/Nu mice. *J Med Virol* 2002; **66**: 70-81
10 **Catalina MD**, Sullivan JL, Bak KR, Luzuriaga K. Differential evolution and stability of epitope-specific CD8(+) T cell responses in EBV infection. *J Immunol* 2001; **167**: 4450-4457
11 **Yuge A**, Kinoshita E, Moriuchi M, Ohno Y, Haga H, Moriuchi H. Persistent hepatitis associated with chronic active Epstein-Barr virus infection. *Pediatr Infect Dis J* 2004; **23**: 74-76
12 **Shibuya A**, Tsuchihashi T, Watanabe M, Nakazawa T, Takeuchi A, Sakurai K, Mitomi H, Saigenji K. Severe chronic active Epstein-Barr virus infection associated with multiple necrotic lesions in the liver. *Hepatol Res* 2003; **25**: 447-454
13 **Ohshima K**, Suzumiya J, Sugihara M, Nagafuchi S, Ohga S, Kikuchi M. Clinicopathological study of severe chronic active Epstein-Barr virus infection that developed in association with lymphoproliferative disorder and/or hemophagocytic syndrome. *Pathol Int* 1998; **48**: 934-943
14 **Fields GB**, Noble RL. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int J Pept Protein Res* 1990; **35**: 161-214
15 **Karnofsky DA**, Abelman WH, Craver LF, Burchenal JH. The use of nitrogen mustards in the palliative treatment of carcinoma. *Cancer* 1948; **1**: 634-656
16 **Sugaya N**, Kimura H, Hara S, Hoshino Y, Kojima S, Morishima T, Tsurumi T, Kuzushima K. Quantitative analysis of Epstein-Barr virus (EBV)-specific CD8+ T cells in patients with chronic active EBV infection. *J Infect Dis* 2004; **190**: 985-988
17 **Maurmann S**, Fricke L, Wagner HJ, Schlenke P, Hennig H, Steinhoff J, Jabs WJ. Molecular parameters for precise diagnosis of asymptomatic Epstein-Barr virus reactivation in healthy carriers. *J Clin Microbiol* 2003; **41**: 5419-5428
18 **Klein E**. The complexity of the Epstein-Barr virus infection in humans. *Pathol Oncol Res* 1998; **4**: 3-7
19 **Nicolas JC**, Maréchal V, Dehée A. [Epstein-Barr virus]. *Bull Acad Natl Med* 1997; **181**: 981-996; discussion 996-997
20 **Sumaya CV**. Endogenous reactivation of Epstein-Barr virus infections. *J Infect Dis* 1977; **135**: 374-379
21 **Zidovec Lepej S**, Vince A, Dakovic Rode O, Remenar A, Jeren T. Increased numbers of CD38 molecules on bright CD8+ T lymphocytes in infectious mononucleosis caused by Epstein-Barr virus infection. *Clin Exp Immunol* 2003; **133**: 384-390
22 **Ishii M**, Yamaguchi N, Ohshima S, Ishii T, Mori KL, Kimura H, Morishima T, Kawase I, Saeki Y. Possibility of preventive treatment for EBV-associated NK cell-lineage proliferative disorders. *Intern Med* 2003; **42**: 250-254
23 **Toyabe S**, Harada W, Uchiyama M. Biclonal expansion of T cells infected with monoclonal Epstein-Barr virus (EBV) in a patient with chronic, active EBV infection. *Clin Exp Immunol* 2003; **134**: 92-97
24 **Gamadia LE**, van Leeuwen EM, Remmerswaal EB, Yong SL, Surachno S, Wertheim-van Dillen PM, Ten Berge IJ, Van Lier RA. The size and phenotype of virus-specific T cell populations is determined by repetitive antigenic stimulation and environmental cytokines. *J Immunol* 2004; **172**: 6107-6114
25 **Papagno L**, Spina CA, Marchant A, Salio M, Rufer N, Little S, Dong T, Chesney G, Waters A, Easterbrook P, Dunbar PR, Shepherd D, Cerundolo V, Emery V, Griffiths P, Conlon C, McMichael AJ, Richman DD, Rowland-Jones SL, Appay V. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol* 2004; **2**: E20
26 **Nociari MM**, Telford W, Russo C. Postthymic development of CD28-CD8+ T cell subset: age-associated expansion and shift from memory to naive phenotype. *J Immunol* 1999; **162**: 3327-3335
27 **Kuijpers TW**, Vossen MT, Gent MR, Davin JC, Roos MT, Wertheim-van Dillen PM, Weel JF, Baars PA, van Lier RA. Frequencies of circulating cytolytic, CD45RA+CD27-, CD8+ T lymphocytes depend on infection with CMV. *J Immunol* 2003; **170**: 4342-4348
28 **Hislop AD**, Annels NE, Gudgeon NH, Leese AM, Rickinson AB. Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J Exp Med* 2002; **195**: 893-905
29 **Ochsenbein AF**, Riddell SR, Brown M, Corey L, Baerlocher GM, Lansdorp PM, Greenberg PD. CD27 expression promotes long-term survival of functional effector-memory CD8+ cytotoxic T lymphocytes in HIV-infected patients. *J Exp Med* 2004; **200**: 1407-1417
30 **Hislop AD**, Gudgeon NH, Callan MF, Fazou C, Hasegawa H, Salmon M, Rickinson AB. EBV-specific CD8+ T cell memory: relationships between epitope specificity, cell phenotype, and immediate effector function. *J Immunol* 2001; **167**: 2019-2029
31 **Tan LC**, Gudgeon N, Annels NE, Hansasuta P, O'Callaghan CA, Rowland-Jones S, McMichael AJ, Rickinson AB, Callan MF. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. *J Immunol* 1999; **162**: 1827-1835
32 **Santolamazza M**, Marinelli RM, Bacosi M, D'Innocenzo S, Miglioresi L, Patrizi F, Delle Monache M, Ricci GL. What kind of hepatitis? *J Int Med Res* 2001; **29**: 441-444
33 **Stránský J**, Benda R, Vodák M, Zikmundová L, Honzáková E. Causes of acute exacerbations of chronic hepatitis B. *Sb Lek* 1995; **96**: 75-83
34 **Sugawara Y**, Mizugaki Y, Uchida T, Torii T, Imai S, Makuuchi M, Takada K. Detection of Epstein-Barr virus (EBV) in hepatocellular carcinoma tissue: a novel EBV latency characterized by the absence of EBV-encoded small RNA expression. *Virology* 1999; **256**: 196-202
35 **Sugawara Y**, Makuuchi M, Takada K. Detection of Epstein-Barr virus DNA in hepatocellular carcinoma tissues from hepatitis C-positive patients. *Scand J Gastroenterol* 2000; **35**: 981-984
36 **Sugawara Y**, Makuuchi M, Kato N, Shimotohno K, Takada K. Enhancement of hepatitis C virus replication by Epstein-Barr virus-encoded nuclear antigen 1. *EMBO J* 1999; **18**: 5755-5760
37 **Li W**, Wu BA, Zeng YM, Chen GC, Li XX, Chen JT, Guo YW, Li MH, Zeng Y. Epstein-Barr virus in hepatocellular carcinogenesis. *World J Gastroenterol* 2004; **10**: 3409-3413
38 **Collin L**, Moulin P, Jungers M, Geubel AP. Epstein-Barr virus (EBV)-induced liver failure in the absence of extensive liver-cell necrosis: a case for cytokine-induced liver dysfunction? *J Hepatol* 2004; **41**: 174-175
39 **Drebber U**, Kasper HU, Krupacz J, Haferkamp K, Kern MA, Steffen HM, Quasdorff M, Zur Hausen A, Odenthal M, Dienes HP. The role of Epstein-Barr virus in acute and chronic hepatitis. *J Hepatol* 2006; **44**: 879-885

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RAPID COMMUNICATION

Association of promoter polymorphism of the CD14 C (-159) T endotoxin receptor gene with chronic hepatitis B

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Abstract

AIM: To investigate whether single-nucleotide polymorphisms in the promoter regions of endotoxin-responsive genes CD14 C (-159) T is associated with chronic hepatitis B.

METHODS: We obtained genomic DNA from 80 patients with established diagnosis of chronic hepatitis B and 126 healthy subjects served as a control population. The CD 14 C (-159) T polymorphism was investigated using an allele specific PCR method.

RESULTS: Twenty seven percent of chronic hepatitis B patients and 75% of controls were heterozygous for CT genotype. The difference between the chronic hepatitis B and control groups was statistically significant [P < 0.0001; Odds ratio (OR) = 2.887; 95% CI: 1.609-5.178]. Twenty four point six percent of chronic hepatitis B and patients 12.3% of the control group were heterozygous for TT genotype. The difference between groups was not statistically significant (P = 0.256; OR = 0.658; 95% CI: 0.319-1.358). Forty eight point four percent of chronic hepatitis B patients and 12.7% of control were homozygote for CC genotype (P < 0.004; OR = 0.416; 95% CI: 0.229-0.755). The frequency of allele C was 61.9% and allele T was 38.1% in hepatitis B patients group. The frequency of allele C was 55.2% and allele T was 44.8% for the control group (P = 0.179; OR = 1.319; 95% CI: 0.881-1.977).

CONCLUSION: The TT heterozygous genotype was not a risk factor for chronic hepatitis B. CC homozygote genotype is protective for hepatitis B. Lack of heterozygosis of genotype CT is a risk factor for chronic hepatitis B. Alleles C or T were not risk factors for chronic hepatitis B.

These findings show the role of a single-nucleotide polymorphism at CD14/-159 on the development of chronic hepatitis B. Endotoxin susceptibility may play a role in the pathogenesis of chronic hepatitis B.

Key words: CD14 C (-159) T gene; Single nucleotide polymorphism; Chronic hepatitis B; Endotoxin susceptibility

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INTRODUCTION

An estimated 350 million persons worldwide are infected with hepatitis B virus (HBV). Hepatitis B carriers are at risk for development of cirrhosis and hepatocellular carcinoma. Persons with chronic hepatitis B infection need life-long monitoring to determine when intervention with antiviral therapy is needed and to observe for serious sequels[1].

The mechanism by which HBV establishes a persistent infection is at present still unclear. Evidence suggests that the clinical manifestations and outcomes after acute liver injury associated with viral hepatitis are determined by the immunologic responses of the host[2]. CD14, a key gene of the innate immune system, functions as a receptor for lipopolysaccharide (LPS), a constitutive element of the bacterial cell wall. CD14 cannot bind to LPS directly. A protein termed LBP (lipopolysaccharide binding protein) must first bind to LPS. The LPS-LBP complex then binds to CD14 and the receptor-ligand complex is internalized. In addition, CD14 is associated with a protein known as Toll-like receptor 4 (TLR-4). As a consequence of the CD14-LPB/LPS interaction at the level of the membrane, TLR-4 becomes activated. TLR-4 plays an important role in signal transduction. Importantly, TLR-4 is now known to activate a transcription factor known as NFκB. Viruses have targeted cellular cytokine production and cytokine receptor-signaling pathways, apoptotic pathways, cell growth and activation pathways, MHC-restricted antigen presentation pathways and humoral immune responses[3].

This study tested whether genetic factors, CD14 C (-159) T, has any role in molecular pathogenesis of chronic

hepatitis B and influences the individual susceptibility for chronic hepatitis B.

MATERIALS AND METHODS

Subjects

We analyzed 80 Iranian patients with chronic hepatitis B and 126 sex-matched control subjects. All chronic hepatitis B patients had visited at a liver clinic in Tehran for regular follow-up examinations. All participants signed the informed consent.

Chronic hepatitis B was based upon biochemical, virologic, histological activity and included patients still on interferron or lamivudine treatment; those who finished treatment course and nonresponders to treatment (lack of virologic and or histologic response by first treatment course in which sustained response was unlikely). Asymptomatic carrier state defined as: chronically HBsAg positive patients who have anti-HBc in serum, anti-HBs is either undetected or detected at low titer against the opposite subtype specificity of the antigen regarded as inactive or asymptomatic carriers, HBV DNA load less than 10^5 copies/mL, HBeAg (+, -), serum liver transaminases of normal range. Inactive hepatitis B surface antigen (HBsAg) carriers were monitored with periodic liver chemistries as liver disease may become active even after many years of quiescence. Controlled subjects had no evidence of hepatitis B infection. The serum of control subjects were evaluated for HBsAg, HBsAb, AST and ALT. Those who had negative HBsAg and HBsAb as well as normal AST and ALT were selected for control groups.

CD14 C (-159) T Genotype Determination

Ten milliliters were collected from each subject into tubes containing 50 mmol/L EDTA, and genomic DNA was isolated from anti-coagulated peripheral blood Buffy coat using Miller's salting-out method[4].

Total genomic DNA from peripheral blood leukocytes was extracted by standard methods. The CD14 C (-159) T polymorphism was investigated using an allele specific PCR method[5]. PCR products were visualized by electrophoresis in 2% (w/v) agarose gel stained with ethidium bromide. The assay thus yields a 381-bp band for T allele and 227-bp band for C allele.

Statistical analysis

The differences in the frequencies of the CD14 genotypes and alleles and other risk factors were analyzed by the χ^2 test. For comparing mean stage of liver pathology in different genotypes, Kruskal-Wallis test was used. Associations and differences with probability value less than 0.05 were considered significant. Statistical data was expressed as mean ± SD. All statistical analyses were performed with the use of SPSS software, version 11.05.

RESULTS

The mean age in hepatitis B group was 36.10 ± 13.78 and in control group was 43.60 ± 16.47. In the hepatitis B group 71.3% of patients were male and 28.8% were female. In the control group 62.7% were male and 37.3% were female. The two groups were matched by sex. In the hepatitis B group, 12% were HBeAg positive, 85% were HBeAg negative, 85% were HBeAb positive and 15% were HBeAb negative. Serum aspartate aminotransfease (AST) levels in 75.9% of hepatitis B patients were < 40 IU/mL and in 24.1% were ⩾ 40 IU/mL. Serum alanine aminotransfease (ALT) in 68.4% of hepatitis B patients was < 40 IU/mL and in 31.6% was ⩾ 40 IU/mL. The state of hepatitis B disease at the time of sampling was as follows: 53.8% were asymptomatic carriers, 31.3% were still on antiviral treatment, 6.3% again returned to treatment, 7.5% were in chronic state and 1.3% were cirrhotics. Liver biopsy was performed in 36 patients of hepatitis B group. Histological classification was measured by Modified Histological activity index (HAI) by Ishak score[6]. Mean stage, grade and pathologic score were 1.85 ± 0.97, 5.28 ± 1.84, and 7.17 ± 2.58, respectively.

Twenty seven percent of hepatitis and 75% of control subjects were heterozygous for CT genotype. The difference between CT genotype was statistically significant (P <0.0001; OR = 2.887; 95% CI: 1.609-5.178). The lack of heterozygosis for genotype CT was a risk factor for hepatitis B. 24.6% of hepatitis and 12.3% of control group subjects were heterozygous for the TT genotype. The difference between groups was not statistically significant (P = 0.256; OR = 0.658; 95% CI: 0.319-1.358). The TT homozygote genotype was not a risk factor for hepatitis B. 48.6% of hepatitis and 12.7% of control group subjects were heterozygous for the CC genotype (P < 0.004; OR = 0.416; 95% CI: 0.229-0.755). The CC homozygote genotype was protective for hepatitis B.

The frequency of allele C was 61.9% and allele T was 38.1% in the hepatitis B group.

The frequency of allele C was 55.2% and allele T was 44.8% for control group (P = 0.179; OR = 1.319; 95% CI: 0.881-1.977). So alleles were not a risk factor for hepatitis B. There were no statistically significant associations between allele frequencies and genotypes frequencies in the hepatitis B group with state of disease; ALT (< 40, ⩾40) IU/mL, mean stage of liver pathology, HBeAg (+, -). Mean stage of liver pathology was not statistically significant in different genotypes (CC, CT, CT) by Kruskal-Wallis test.

DISCUSSION

In a previous study[7], the effect of recombinant HBsAg (rHBsAg) on LPS- and IL-2-induced activation of monocytes was investigated. It showed that recombinant HBsAg particles, which contain the S protein only, bind almost exclusively to monocytes. Further it showed that recombinant HBsAg (rHBsAg) particles not only inhibit LPS-induced secretion of IL-1β and TNFα, but also inhibit IL-2-induced secretion of IL-8. Their results suggested that monocytes express a receptor that is recognized by HBsAg and that HBV produces HBsAg in excess amounts to interfere with the normal function of antigen-presenting cells.

In our population, HBeAg negative chronic hepatitis B is more common than HBeAg positive. But difference

in associations of frequency of alleles and genotypes in HBeAg negative versus positive chronic hepatitis B patients were not statistically significant. Besides the viral role, we aimed to investigate the CD14 C (-159) T polymorphism as a host factor, which deteriorates the hepatitis course and outcome in our population.

The CD14 promoter genotype may affect inflammatory processes and be involved in atherogenesis, and it is therefore possible that this genotype might also be associated with other major forms of thrombotic disease, such as ischemic cerebrovascular disease, coronary artery disease. LPS is a structural component of gram-negative bacteria and is bound in plasma by the LPS binding protein[8]. The LPS binding protein complex then binds to a glycosylphosphatidylinositol-anchored membrane protein, membranous CD14 (mCD14), on monocytes and macrophages and activates these cells. The activated phagocytes in turn secrete inflammatory cytokines through which LPS indirectly activates endothelial cells. Soluble CD14 (sCD14), which lacks a glycosylphosphatidylinositol anchor, can also be found in plasma. Endothelial cells and smooth muscle cells, lacking their own mCD14, are directly activated by LPS-sCD14 complex[9,10]. Directly and indirectly activated endothelial cells express cell adhesion molecules and increased procoagulant activity, and they release free radicals, thereby mediating the initiation and development of atherosclerosis.

A previous study[11], demonstrated T allele frequency was significantly higher in myocardial infarction survivors and that the density of monocyte mCD14 was higher in T/T homozygotes than in other genotypes.

In another study[12], the possible association between the C (-260)→T polymorphism in the CD14 promoter and the occurrence of symptomatic ischemic cerebrovascular disease (CVD) was tested. They concluded that the C (-260)→T polymorphism in the CD14 promoter is not associated with an increased risk for CVD.

A previous study[13] mentioned activated Kupffer cells release proinflammatory cytokines, a process that is regulated by the CD14 endotoxin receptor (CD14). Also, both clinical and experimental data suggest that Kupffer cell activation by gut-derived endotoxins and other bacterial products is an important pathogenic factor. In that study, the association of CD14 promoter polymorphism with different forms of alcoholic liver damage (ALD) was examined in 3 separate autopsy series. The overall age-adjusted risk for cirrhosis was 3.08 for the carriers of the CT genotype, and 4.17 for the homozygous TT genotype. Their results suggested that in the relatively isolated Finnish population, the T allele and in particular, TT homozygotes confers increased risk of alcoholic liver damage and are at a high risk to develop cirrhosis.

Another study[14] investigated whether single-nucleotide polymorphisms in the promoter regions of endotoxin-responsive genes CD14 and tumor necrosis factor-α (TNF-α) were associated with biliary atresia (BA) and idiopathic neonatal cholestasis (INC) in 90 patients with established diagnosis of BA and 28 patients with INC. Also, forty-two adult patients with hepatitis B-related cirrhosis and 143 healthy children served as control populations. According to that study the single-nucleotide polymorphism at CD14/-159 is associated with the development of BA and INC. They concluded that endotoxin susceptibility might play a role in the pathogenesis of infantile cholestasis.

In the present study, we did not compare risk factors of hepatitis B infection, its routs of exposure and transmission in hepatitis B and control groups. Although determining the exact time of hepatitis B infection was impossible. Whether specific polymorphisms of CD14 C (-159) T has any correlation with progression of liver pathology or whether it indirectly produces needs further study and a larger sample size.

CONCLUSIONS

Our study demonstrated that the role for CD14 C (-159) T polymorphism on the development of chronic hepatitis B. Although the number of subjects in the chronic hepatitis B subtypes was relatively small, differences in the genotype distributions for chronic hepatitis B subtypes was significant.

Lack of heterozygosis of genotype CT is a risk factor for chronic hepatitis B.

TT homozygote genotype was not a risk factor for chronic hepatitis B. CC homozygote genotype is protective for chronic hepatitis B.

These findings show a role for single-nucleotide polymorphisms at CD14/-159 C (-159) T on development of chronic hepatitis B. Endotoxin susceptibility may play a role in the pathogenesis of chronic hepatitis B.

REFERENCES

1 **Lok AS**, McMahon BJ. Chronic hepatitis B: update of recommendations. *Hepatology* 2004; **39**: 857-861

2 **Dienstag JL**, Isselbacher KJ. Acute viral hepatitis. In: Kasper LK, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL. Harrison's Principles of Internal Medicine. 16th ed. New York: MacGraw-Hill, 2005: 1834-1930

3 **Tortorella D**, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral subversion of the immune system. *Annu Rev Immunol* 2000; **18**: 861-926

4 **Sambrook J**, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor, 1989: 443-454

5 **Karhukorpi J**, Ikäheimo I, Karvonen J, Karttunen R. Promoter region polymorphism of the CD14 gene (C-159T) is not associated with psoriasis vulgaris. *Eur J Immunogenet* 2002; **29**: 57-60

6 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293

7 **Vanlandschoot P**, Van Houtte F, Roobrouck A, Farhoudi A, Leroux-Roels G. Hepatitis B virus surface antigen suppresses the activation of monocytes through interaction with a serum protein and a monocyte-specific receptor. *J Gen Virol* 2002; **83**: 1281-1289

8 **Wright SD**, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; **249**: 1431-1433

9 **Frey EA**, Miller DS, Jahr TG, Sundan A, Bazil V, Espevik T, Finlay BB, Wright SD. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J Exp Med* 1992; **176**: 1665-1671

10 **Loppnow H**, Stelter F, Schönbeck U, Schlüter C, Ernst M, Schütt C, Flad HD. Endotoxin activates human vascular smooth muscle cells despite lack of expression of CD14 mRNA

or endogenous membrane CD14. *Infect Immun* 1995; **63**: 1020-1026

11 **Hubacek JA**, Rothe G, Pit'ha J, Skodová Z, Staněk V, Poledne R, Schmitz G. C(-260)-->T polymorphism in the promoter of the CD14 monocyte receptor gene as a risk factor for myocardial infarction. *Circulation* 1999; **99**: 3218-3220

12 **Ito D**, Murata M, Tanahashi N, Sato H, Sonoda A, Saito I, Watanabe K, Fukuuchi Y. Polymorphism in the promoter of lipopolysaccharide receptor CD14 and ischemic cerebrovascular disease. *Stroke* 2000; **31**: 2661-2664

13 **Järveläinen HA**, Orpana A, Perola M, Savolainen VT, Karhunen PJ, Lindros KO. Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease. *Hepatology* 2001; **33**: 1148-1153

14 **Shih HH**, Lin TM, Chuang JH, Eng HL, Juo SH, Huang FC, Chen CL, Chen HL. Promoter polymorphism of the CD14 endotoxin receptor gene is associated with biliary atresia and idiopathic neonatal cholestasis. *Pediatrics* 2005; **116**: 437-441

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RAPID COMMUNICATION

Inhibition of hepatitis B virus production by *Boehmeria nivea* root extract in HepG2 2.2.15 cells

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Abstract

AIM: To explore the anti-hepatitis B virus (HBV) effects of Boehmeria nivea (B. nivea) root extract (BNE) by using the HepG2 2.2.15 cell model system.

METHODS: Hepatitis B surface antigen (HBsAg), hepatitis B virus e antigen (HBeAg), and HBV DNA were measured by using ELISA and real-time PCR, respectively. Viral DNA replication and RNA expression were determined by using Southern and Northern blot, respectively.

RESULTS: In HepG2 2.2.15 cells, HBeAg (60%, $P < 0.01$) and particle-associated HBV DNA (> 99%, $P < 0.01$) secretion into supernatant were significantly inhibited by BNE at a dose of 100 mg/L, whereas the HBsAg was not inhibited. With different doses of BNE, the reduced HBeAg was correlated with the inhibition of HBV DNA. The anti-HBV effect of BNE was not caused by its cytotoxicity to cells or inhibition of viral DNA replication and RNA expression.

CONCLUSION: BNE could effectively reduce the HBV production and its anti-HBV machinery might differ from the nucleoside analogues.

Key words: *Boehmeria nivea*; Medicinal herb; Antiviral agent; Hepatitis B virus; Anti-hepatitis B virus; HepG2 2.2.15

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INTRODUCTION

Hepatitis B virus (HBV) has infected more than 4 million people worldwide. About 20% of patients infected with HBV may lead to chronic hepatitis, liver cirrhosis, and hepatocarcinoma. HBV, belonging to the *Hepadnaviridae* family, is a non-cytopathic DNA virus with an icosahedral capsid that replicates *via* reverse transcription of an RNA intermediate[1]. Although the molecular biology aspects of the HBV genome have been described in detail, the mechanisms of viral packaging and transport remain to be elucidated[2]. Nevertheless, HBV-DNA-transfected cells and virus infection animal models have aided the efforts to reveal the mechanism behind the HBV replication cycle. These results led to the identification of the first antiviral agents targeting the reverse transcription process[3].

Chronic hepatitis type-B patients are clinically treated with interferon alpha (INF-α) and nucleoside analogue lamivudine (3TC), adefovir or entecavir[4,5], which are analogues of reverse-transcriptase inhibitors[6,7]. INF-α inhibits viral replication and acts as an immuno-modulator. However, its disadvantages include limited effectiveness (40% response rate)[8], low efficacy with respect to cost, and serious side effects. For 3TC, its inhibition is reversible, and continuous treatment often leads to the development of drug-resistant HBV variants (70% of patients after 4 years of treatment)[9]. Both adefovir and entecavir are used against 3TC-resistant viruses; however, resistances to these two drugs have been reported in lamivudine-resistant patients[10-12]. The use of combination therapy, such as INF-α plus 3TC, 3TC plus adefovir and 3TC plus entecavir, may yield additive or synergistic effects or reduce the emergence of resistance, though serious side effects and unsatisfactory efficacy still present problems. Undeniably, there is a demand for new and improved therapies.

The large repertoire of herbal compounds may show potential in developing new ways to combat previously considered "incurable" diseases, provided that these compounds (or often, mixtures of compounds) could satisfy current government regulations. At present, alternative or traditional medical resources are used by more than 80% of the population in developing countries and by an increasing number of people in other parts of

the globe[13,14]. Complementary and alternative therapies for chronic hepatitis are also intensively explored and the results appear promising[15]. Patients with chronic liver diseases are treated with some medicinal herbs exhibiting strong anti-viral activities[16], including daphnoretin from *Wikstroemia indica*[17], costunolide and dehydrocostus lactone from *Saussurea lappa* Clarks[18], osthole from *Angelica pubescens*[19], and the extracts of genus *Phyllanthus* of the *Euphobiaceae* family[20]. Furthermore, genus *Phyllanthus* exhibited a positive effect on the clearance of serum HBsAg in clinical trials conducted on chronic HBV infections, and a synergistic effect when administered with IFN-α[20]. *B. nivea* has been distributed and used therapeutically in China and Taiwan for diuretic, antipyretic, and hepatoprotective purposes. Recently, it has been reported that root extracts of *B. nivea* exhibited hepatoprotective activities against CCl_4-induced liver injuries, and anti-oxidant effects on $FeCl_2$-ascorbate-induced lipid peroxidation in rat liver homogenate[21].

To investigate the anti-viral mechanism of *B. nivea* extract (BNE), HBV-producing hepatoma HepG2 2.2.15 cells, which secrete HBsAg, HBeAg and complete Dane particles[22], were chosen for the evaluation of the anti-HBV effect of BNE. Here, we assess anti-HBV activities of BNE by measuring HBsAg, HBeAg, HBV DNA in supernatant, and replication intermediate HBV DNA and HBV RNA within the cells.

MATERIALS AND METHODS

Preparation of BNE

To prepare the *B. nivea* plant extract utilized in our experiments, the roots of the plants were collected and dried. One hundred gram of the dried roots was cut into pieces approximately 0.5 cm in length before boiling them in 1 L of 200 mL/L ethanol (1:10 ratio) under reflux for 3 h. The decoction was filtered through a 0.22-μm filter and lyophilized. The lyophilized powder was dissolved in normal PBS and adjusted to stock concentration (100 g/L) prior to application to the cells.

Cell lines and culture

The HepG2 2.2.15 cell line was kindly provided by Dr. Ho MS (Academia Sinica, Taipei, Taiwan, China), and Human hepatoma HepG2 cells were obtained from American Type Culture Collection (ATCC). These cells were maintained in MEM (Eagle) plus 100 mL/L fetal bovine serum (FBS) supplement with 1.5 g/L sodium bicarbonate, 0.1 mmol/L non-essential amino acids, 1.0 mmol/L sodium pyruvate, and 100 units/mL penicillin G and 100 mg/L streptomycin. A final concentration of 200 mg/L G418 was contained in the medium for the maintenance of HepG2 2.2.15 cells. Before the experiment, the cell count was adjusted to 1×10^6/mL, and cell viability to higher than 85% by trypan blue exclusion test.

Drug treatment protocols

For drug treatment, 1×10^5 cells of HepG2 2.2.15 were seeded in a 12-well plate and allowed to grow for 3 d before treatment with different concentrations of BNE. Cells were refed with drug-containing fresh medium every 3 d for up to 12 d in time-dependent experiment. In Southern and Northern blot assay, the culture condition was the same as described above (10 mg/L 3TC were also added), and in which time of drug treatments were only for 1 d.

Analysis of cytotoxicity

HepG2 2.2.15 cells were used for determining cytotoxicity of BNE. Cells were inoculated onto a 96-well plate at a density of 1×10^4 cells per 100 μL prior to drug treatment. BNE was added at concentrations of 0.1, 1, 10, and 100 mg/L and the cells were refed with drug-containing fresh medium every 3 d for up to 12 d. After drug treatment, the cytotoxicity was measured based on the reduction of MTT (Sigma, St. Louis, USA) in mitochondria[23].

Determination of HBsAg and HBeAg levels by ELISA

Conditioned medium was collected and the HBsAg and HBeAg levels were determined semi-quantitatively using ELISA assay [SURASE B-96 (TMB), General Biologicals corp., Taiwan, China] according to the manufacturer's instructions.

Supernatant HBV DNA extraction and analysis by quantitative real-time PCR

The supernatant HBV DNA was extracted from conditioned medium as previously described[24] and stored in -20℃ prior to real-time PCR analysis. The quantity of HBV DNA in culture medium was quantified with the ABI 7500 Sequence Detection System by using HBV RealQuant PCR kit (General Biologicals Corp., Taiwan, China) according to the manufacturer's instructions. Briefly, the PCR programming was performed with an initial denaturing steps at 50℃ for 2 min and 95℃ for 10 min, followed by 45 amplification cycles at 95℃ for 15 s and annealing/extending at 58℃ for 1 min.

The 50% effective concentration (EC_{50}), defined as the drug concentration that reduces the level of HBV DNA in the culture medium by 50%, was calculated by four parameter logistic curve equation.

Southern and Northern blot detection of HBV DNA and mRNA in HepG2.2.15 cell

HepG2 2.2.15 cells were cultured in MEM medium and treated with 0.1, 1, and 10 mg/L BNE for 1 d. Cells were lysed with 0.8 mL of 0.01 mol/L Tris-HCl (pH 8.0), 0.05 mol/L NaCl, 5 mL/L NP-40, 1 mmol/L EDTA at room temperature for 10 min as previously described[25]. For the Southern hybridizations, 20 μg of total DNA was digested with *Hin*dⅢ, electrophoresed on a 14 g/L agarose gel, and then transferred to nylon membrane. The probe for hybridization was synthesized from PCR amplification of a plasmid containing the full-length HBV genome (kindly provided by Dr. Ho MS, Academia Sinica, Taipei, Taiwan, China), and then the probe was labeled with digoxigenin-dUTP (DIG) by the DIG-high Prime DNA labeling and Detection Starter kitⅡ according to the manufacturer's protocol (Roche, Basel, Switzerland). The membrane was hybridized with HBV probe at 50℃ overnight. For Northern blot analysis, total RNA was isolated from BNE-treated and untreated HepG2 2.2.15 cells by using the TRIZOL kit (Invitrogen, CA, USA). A total of 20 μg of RNA was resolved in 12 g/L denatured gel and then

transferred onto the nylon membrane and the membrane was hybridized with DIG-labeled HBV DNA fragment described above. For hybridization of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the full-length HBV DNA probe was removed from the membrane by washing twice at 37℃ in 0.2 mol/L NaOH containing 1 mL/L sodium dodecyl sulfate (SDS) solution for 15 min and then re-hybridized with DIG-labeled probe for G3PDH.

Statistical analysis

Data were expressed as mean ± SE of three independent experiments. Statistical analysis was performed using Student's *t*-test. A P value less than 0.05 was considered statistically significant.

RESULTS

Effects of BNE on secreted HBsAg, HBeAg and HBV DNA from HepG2 2.2.15 cultures

Anti-HBV activity of BNE was investigated by using HepG2 2.215 cells, which can secrete HBV particles. When HepG2 2.2.15 cells were treated with various concentrations of BNE, secretion of HBeAg, but not HBsAg, was significantly suppressed compared to vehicle controls (Figure 1A and B). The suppression of HBeAg was dose-dependent and approximately 20% of inhibition was observed in cells treated with 10 mg/L BNE. Moreover, a significant suppression of HBeAg secretion (approximately 60%) was observed in cells treated with 100 mg/L BNE (Figure 1B).

We also measured the amount of viral DNA secreted in the medium (supernatant HBV DNA) by using real-time PCR. As shown in Figure 1C, supernatant HBV DNA was dramatically decreased compared to vehicle control, since the first day of treatment, in 10 and 100 mg/L BNE-treated culture medium. Although the amounts of supernatant HBV DNA increased while cells continued to grow during the experiment, approximately 95% inhibition of DNA secretion was observed in the 12-d culture treated with BNE (10 mg/L) (Figure 1C). Notably, these results showed that the production of HBsAg and HBeAg was only slightly suppressed by 10 mg/L BNE, but supernatant HBV DNA levels were dramatically suppressed by BNE at the same dose.

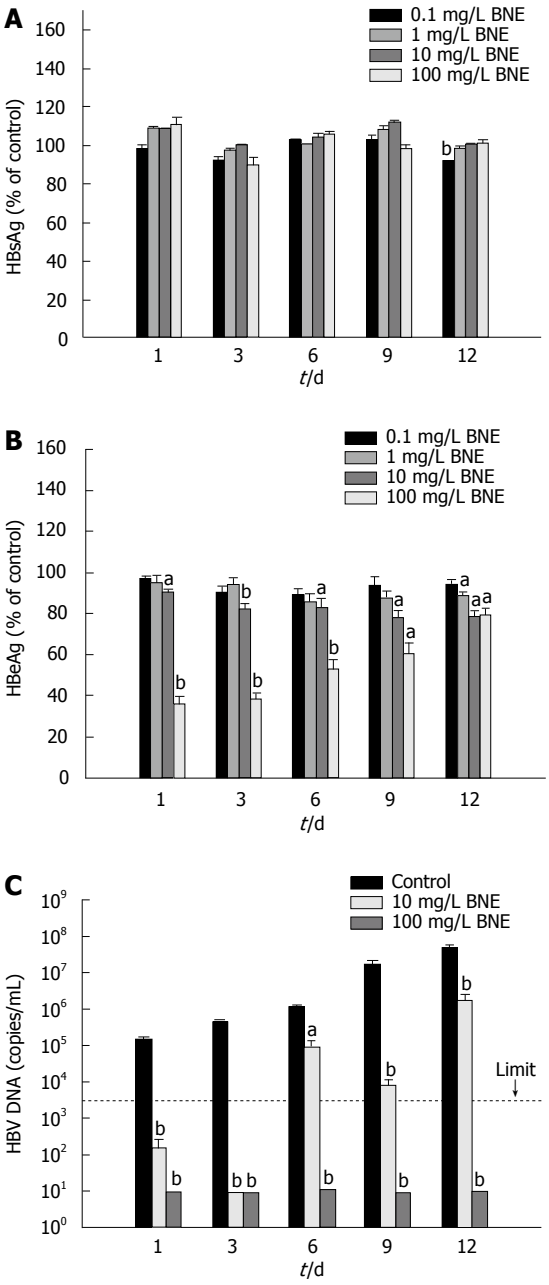

Figure 1 Effects of BNE on secreted HBsAg, HBeAg and HBV DNA from HepG2 2.2.15 cell cultures. **A**: HBsAg level; **B**: HBeAg level; **C**: Viral DNA. The dotted line presents the limitation of this kit (3000 copies/mL). Data are expressed as mean ± SE of three independent experiments. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* the corresponding controls (Student's *t*-test).

Cytotoxic effects of BNE on HepG2 2.2.15 cells

Suppression of HBV production by BNE might be a result of its cytotoxicity, and this possibility was examined in HepG2 2.2.15 cells by using MTT assay. No apparent cytotoxicity was detected in HepG2 2.2.15 cells up to 12 d after exposure to BNE at different concentrations (0.1, 1, 10 and 100 mg/L) (Figure 2), suggesting that the suppression of supernatant viral DNA levels by BNE was not caused by its cytotoxicity.

Determination of the effective concentration of BNE anti-HBV activity

The effective concentration of BNE to suppress 50% of secreted HBV DNA (EC_{50}) was determined with various concentrations of BNE on d 1. The suppression of secreted HBV DNA was shown in a dose-dependent manner with BNE treatment (70% at 0.1 mg/L and 100% at ⩾ 10 mg/L) (Figure 3); accordingly, the EC_{50} was 0.0462 mg/L. Here, the concentration of BNE (10 mg/L) used for the study of anti-HBV activity was about 200-fold of EC_{50}.

Effects of BNE on intracellular HBV DNA replication and transcription in HepG2 2.2.15 cells

To address the mechanism of inhibition by BNE in HepG2 2.2.15 cells, we analyzed the viral mRNA after 24 h exposure to BNE by Northern blot using full-length HBV genome as a probe (Figure 4A) and intracellular relaxed circular (RC) and single-stranded (SS) forms of HBV DNA by Southern blot in parallel (Figure 4B). The levels

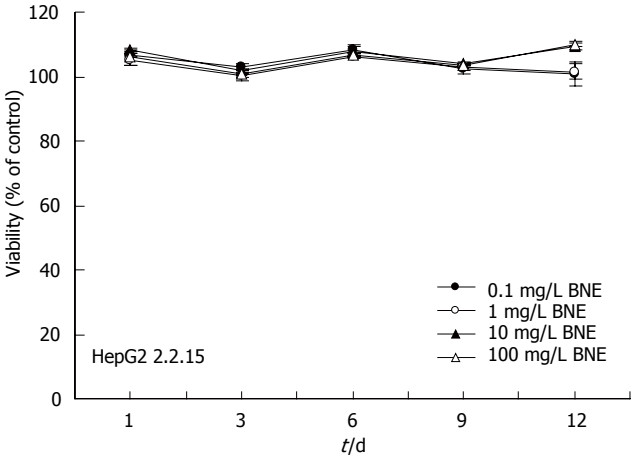

Figure 2 Cytotoxic effects of BNE on HepG2 2.2.15 cells. Data are expressed as mean ± SE of three independent experiments (MTT assay).

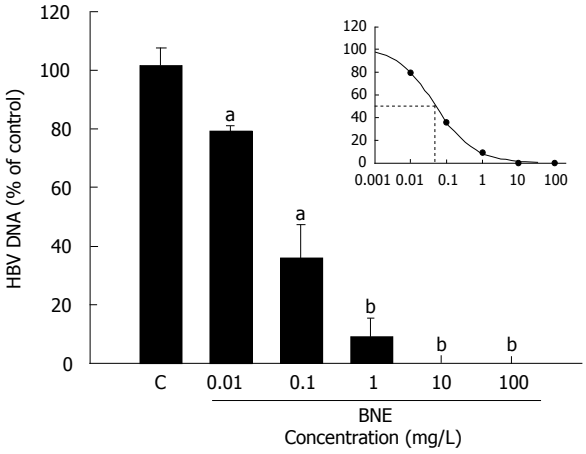

Figure 3 Determination of the effective concentration of BNE anti-HBV activity. Cells were treated for 24 h. Data are expressed as mean ± SE of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the corresponding controls (Student's *t*-test).

of viral mRNA were not affected in either BNE-treated or 3TC-treated cells (Figure 4A). Moreover, Southern blot results indicated that BNE did not suppress intracellular RC and SS forms of HBV DNA. In Contrast, an apparent inhibition of intracellular RC and SS forms of HBV DNA was observed in 3TC-treated cells, which was significantly different from the BNE group (Figure 4B). These results suggested that BNE did not apparently decrease viral DNA replication and viral mRNA expression in HepG2 2.2.15 cells, and that the anti-HBV mechanism of BNE seemed to be different from that of 3TC.

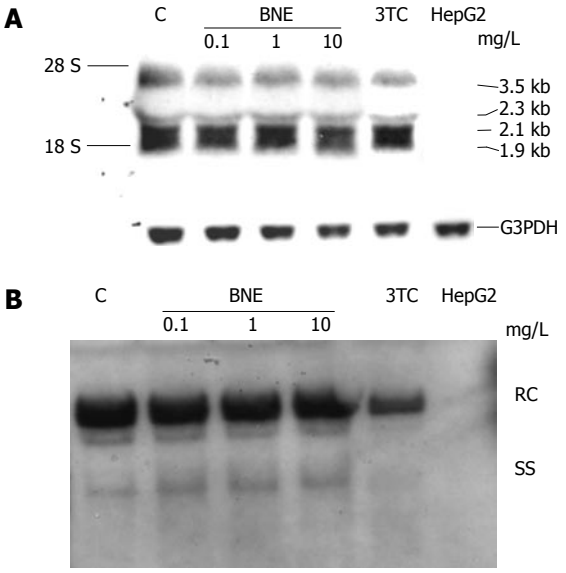

Figure 4 Effects of BNE on intracellular HBV DNA replication and transcription in HepG2 2.2.15 cells. Cells were treated for 1 d. **A**: Northern analysis; **B**: Southern analysis.

DISCUSSION

In this study, we first demonstrated that BNE had anti-HBV activity of inhibiting the supernatant HBV DNA levels in a dose-dependent manner in HepG2 2.2.15 cells without blocking HBsAg secretion. This inhibition was caused neither by the toxicity of BNE to HepG2 2.2.15 cells, nor by blocking HBV gene expression and replication. The significant inhibition of supernatant HBV DNA levels was observed at a concentration greater than 10 mg/L of BNE in HepG2 2.2.15 cells. In addition, BNE could also dose-dependently inhibit the secretion of HBeAg. Although BNE had higher inhibitory ability on HBeAg secretion at 100 mg/L than that at 10 mg/L, it still could efficiently inhibit secreted HBV DNA at the latter concentration. This result might be due to the fact that BNE comprise multiple compounds, and the effective concentration of those compounds to inhibit secreted HBV DNA was lower than that of the compounds required for inhibition of HBeAg.

Currently, 3TC and other nucleoside analogues have been shown to inhibit HBV replication both *in vitro* and *in vivo*[24,26,27]. A previous study had reported that 3TC, a viral polymerase inhibitor, reduced episomal DNA (RC and SS form), whereas HBV-specific RNAs were not affected in HepG2 2.2.15 cells[24]. In this study, we could not find any apparent reduction of HBV-specific RNAs or intracellular SS and RC DNA in response to BNE, and that these data revealed that the anti-HBV mechanism of BNE might be different from 3TC that targeted the viral polymerase. Since the HBV mRNAs were transcribed from the integrated DNA, it was not unexpected that HBV-specific transcripts were not affected by BNE treatment. The finding that supernatant HBV DNA rather than HBsAg and HBeAg was dramatically inhibited by 10 mg/L BNE after 24-h treatment might come from the possibility that exported virions have outer protein coats or HBsAg without packaging DNA. Though the mechanism of anti-viral effects by BNE remains unclear, we deduced that BNE might block coating and secretion of HBV containing nucleocapsids or destabilize HBV DNA containing nucleocapsids.

Whether the HBV-inhibiting effects of BNE could be contributed to a single component, or multiple components, is currently unknown. In order to explore the active compound for anti-HBV activity, we have attempted to analyze the chemical composition of BNE by HPLC. Very

little, if any, nucleotide analogues were detected (data not shown). Fractionation experiments of BNE are currently being executed in our laboratory. Recently, Herteroaryldihy dropyrimidines (HAP)[28], Bis-ANS[29], and alkylated imino sugars[30,31] have been found to block the viral production by interference with either nucleocapsid assembly or nucleocapsid maturation, and these compounds might have the potential to be developed as non-viral polymerase targeting antiviral drugs.

In conclusion, the BNE, together with other medicinal herbs that exploit different action modes to inhibit HBV, could be administered in combination with other polymerase inhibitors or cytokines, providing possibly a novel HBV treatment strategy to the current therapies.

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REFERENCES

1 **Ganem D**, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-1129
2 **Knipe DM**, Howley PM. Fundamental virology. 4th ed. Philadelphia: Lippincott Williams & Wilkins, 2001
3 **Summers J**, Mason WS. Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. *Cell* 1982; **29**: 403-415
4 **Lai CL**, Rosmawati M, Lao J, Van Vlierberghe H, Anderson FH, Thomas N, Dehertogh D. Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. *Gastroenterology* 2002; **123**: 1831-1838
5 **Chang TT**, Gish RG, Hadziyannis SJ, Cianciara J, Rizzetto M, Schiff ER, Pastore G, Bacon BR, Poynard T, Joshi S, Klesczewski KS, Thiry A, Rose RE, Colonno RJ, Hindes RG. A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198-1209
6 **Buster EH**, Janssen HL. Antiviral treatment for chronic hepatitis B virus infection--immune modulation or viral suppression? *Neth J Med* 2006; **64**: 175-185
7 **Thomas H**, Foster G, Platis D. Mechanisms of action of interferon and nucleoside analogues. *J Hepatol* 2003; **39** Suppl 1: S93-S98
8 **Niederau C**, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, Häussinger D. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. *N Engl J Med* 1996; **334**: 1422-1427
9 **Fischer KP**, Gutfreund KS, Tyrrell DL. Lamivudine resistance in hepatitis B: mechanisms and clinical implications. *Drug Resist Updat* 2001; **4**: 118-128
10 **Angus P**, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, Brosgart C, Colledge D, Edwards R, Ayres A, Bartholomeusz A, Locarnini S. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology* 2003; **125**: 292-297
11 **Fung SK**, Chae HB, Fontana RJ, Conjeevaram H, Marrero J, Oberhelman K, Hussain M, Lok AS. Virologic response and resistance to adefovir in patients with chronic hepatitis B. *J Hepatol* 2006; **44**: 283-290
12 **Tenney DJ**, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, Plym M, Pokornowski K, Yu CF, Angus P, Ayres A, Bartholomeusz A, Sievert W, Thompson G, Warner N, Locarnini S, Colonno RJ. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob Agents Chemother* 2004; **48**: 3498-3507
13 **Gesler WM**. Therapeutic landscapes: medical issues in light of the new cultural geography. *Soc Sci Med* 1992; **34**: 735-746
14 **Bent S**, Ko R. Commonly used herbal medicines in the United States: a review. *Am J Med* 2004; **116**: 478-485
15 **Coon JT**, Ernst E. Complementary and alternative therapies in the treatment of chronic hepatitis C: a systematic review. *J Hepatol* 2004; **40**: 491-500
16 **Strader DB**, Bacon BR, Lindsay KL, La Brecque DR, Morgan T, Wright EC, Allen J, Khokar MF, Hoofnagle JH, Seeff LB. Use of complementary and alternative medicine in patients with liver disease. *Am J Gastroenterol* 2002; **97**: 2391-2397
17 **Chen HC**, Chou CK, Kuo YH, Yeh SF. Identification of a protein kinase C (PKC) activator, daphnoretin, that suppresses hepatitis B virus gene expression in human hepatoma cells. *Biochem Pharmacol* 1996; **52**: 1025-1032
18 **Chen HC**, Chou CK, Lee SD, Wang JC, Yeh SF. Active compounds from Saussurea lappa Clarks that suppress hepatitis B virus surface antigen gene expression in human hepatoma cells. *Antiviral Res* 1995; **27**: 99-109
19 **Huang RL**, Chen CC, Huang YL, Hsieh DJ, Hu CP, Chen CF, Chang C. Osthole increases glycosylation of hepatitis B surface antigen and suppresses the secretion of hepatitis B virus *in vitro*. *Hepatology* 1996; **24**: 508-515
20 **Liu J**, Lin H, McIntosh H. Genus Phyllanthus for chronic hepatitis B virus infection: a systematic review. *J Viral Hepat* 2001; **8**: 358-366
21 **Lin CC**, Yen MH, Lo TS, Lin JM. Evaluation of the hepatoprotective and antioxidant activity of Boehmeria nivea var. nivea and B. nivea var. tenacissima. *J Ethnopharmacol* 1998; **60**: 9-17
22 **Sells MA**, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA* 1987; **84**: 1005-1009
23 **Carmichael J**, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res* 1987; **47**: 943-946
24 **Doong SL**, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus *in vitro* by 2',3'-dideoxy-3'-thiacytidine and related analogues. *Proc Natl Acad Sci USA* 1991; **88**: 8495-8499
25 **Lu X**, Hazboun T, Block T. Limited proteolysis induces woodchuck hepatitis virus infectivity for human HepG2 cells. *Virus Res* 2001; **73**: 27-40
26 **Dienstag JL**, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 1995; **333**: 1657-1661
27 **Dixon JS**, Boehme RE. Lamivudine for the treatment of chronic hepatitis B. *Acta Gastroenterol Belg* 2000; **63**: 348-356
28 **Weber O**, Schlemmer KH, Hartmann E, Hagelschuer I, Paessens A, Graef E, Deres K, Goldmann S, Niewoehner U, Stoltefuss J, Haebich D, Ruebsamen-Waigmann H, Wohlfeil S. Inhibition of human hepatitis B virus (HBV) by a novel non-nucleosidic compound in a transgenic mouse model. *Antiviral Res* 2002; **54**: 69-78
29 **Zlotnick A**, Ceres P, Singh S, Johnson JM. A small molecule inhibits and misdirects assembly of hepatitis B virus capsids. *J Virol* 2002; **76**: 4848-4854
30 **Lu X,** Tran T, Simsek E, Block TM. The alkylated imino sugar, n-(n-Nonyl)-deoxygalactonojirimycin, reduces the amount of hepatitis B virus nucleocapsid in tissue culture. *J Virol* 2003; **77**: 11933-11940
31 **Deres K**, Schröder CH, Paessens A, Goldmann S, Hacker HJ, Weber O, Krämer T, Niewöhner U, Pleiss U, Stoltefuss J, Graef E, Koletzki D, Masantschek RN, Reimann A, Jaeger R, Gross R, Beckermann B, Schlemmer KH, Haebich D, Rübsamen-Waigmann H. Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. *Science* 2003; **299**: 893-896

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RAPID COMMUNICATION

Resection of non-cystic adenocarcinoma in pancreatic body and tail

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Abstract

AIM: To report the outcome of Chinese patents with non-cystic adenocarcinoma in pancreatic body and tail (NCAPBT) after resection and to discuss its surgical strategy.

METHODS: Resection of NCAPBT was performed in eight Chinese patients with complete clinical-pathological data in our hospital from January 2000 to May 2004. The surgical strategy was explored by analyzing the results of these patients.

RESULTS: The resection rate of NCAPBT in patients without back pain was higher than that in patients with back pain (66.67% *vs* 20%, 2/3 *vs* 1/5). The prognosis in the group receiving palliative resection was poorer than that in the group receiving curative resection. The median survival time was 12 mo in the curative resection group and 6 mo in the palliative resection group, respectively.

CONCLUSION: The overall survival time of the Chinese patients with NCAPBT is dismal. The Chinese patients after curative resection of NCAPBT have a longer survival time. The Chinese NCAPBT patients with back pain trend to have a lower curative resection rate, but back pain should not be considered a contraindication for curative resection.

Key words: Pancreas; Adenocarcinoma; Pancreatectomy; Survival; Back bain

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INTRODUCTION

The prognosis of pancreatic body and tail cancer is dismal[1-10]. Its curative resection is rarely performed, only a few reports are available[4-10]. The outcome of 8 Chinese patients with non-cystic adenocarcinoma in pancreatic body and tail (NCAPBT) after resection is reported and the clinical-pathological characteristics and surgical strategy of NCAPBT are discussed in this paper.

MATERIALS AND METHODS

Resection of NCAPBT was performed in eight Chinese patients with complete clinical-pathological data from January 2000 to September 2004 in our hospital. The outcome of all the patients is summarized in Tables 1 and 2. The average age of the patients was 67 years (range, 53-79 years) and the ratio of male to female was 1:1. Abdominal pain was found in 87.5%, weight loss in 75% and back pain in 62.5% patients, respectively. No patient had jaundice. The mean tumor size was 5.04 cm (range, 2.3-8 cm). Curative distal pancreatectomy and splenectomy were performed in 3 patients, palliative distal pancreatectomy and splenectomy in 4 patients, palliative segmental pancreatectomy in 1 patient. Curative resection was defined as resection with all gross tumor tissues removed and negative microscopic margins. Resection with gross residual tumor and/or distal metastasis was considered palliative resection. All the 8 patients had histology-verified NCAPBT. Seven of 8 NCAPBTs were ductal adenocarcinoma. No.4 NCAPBT was mucinous non-cystic adenocarcinoma (Tables 1 and 2). No cystic adenocarcinoma and other rare adenocarcinomas such as adenosquamous carcinoma, undifferentiated adenocarcinoma and primary cancer were found. All the 8 patients were followed up. The log-rank test was used for comparison of differences in survival. Chi-square test or Fisher's exact test was used to evaluate the correlation between categorical data. $P < 0.05$ was considered statistically significant.

RESULTS

The outcome of the 8 patients is summarized in Tables 1 and 2. Intra-abdominal abscess was found in 1 patient and no major postoperative complications were found in

Table 1 Clinical data of NCAPBT

Patient No.	Gender	Age (yr)	Abdominal pain	Back pain	Weight loss (kg)	Location	Size (cm)	Survival (mo)
1	M	79	Y	Y	NA	BT	5	6
2	F	62	Y	N	5	BT	5	6
3	F	66	Y	Y	7.5	BT	6	4
4	M	65	Y	Y	NA	B	5	8
5	M	53	Y	Y	5	BT	NA	6
6	F	71	Y	N	4.5	B	2.3	12
7	F	68	Y	Y	5	BT	4	28
8	M	72	N	N	3	T	8	8

NA: Not available; BT: Pancreatic body and tail; B: Pancreatic body; T: Pancreatic tail; Y: Yes; N: No.

Table 2 Data of surgery and pathology of NCAPBT

Patient No.	pT	pN	pM	Histological differentiation	Resection	Blood loss (mL)	Operative duration (min)	Reason for non-curative resection
1	4	x	0	Moderate	Palliative	600	140	Invasion of super mesenteric artery
2	4	1	0	Well	Palliative	600	235	Adhesion of abdominal aorta
3	4	x	0	Well	Palliative	200	110	Invasion of celiac trunk and abdominal aorta
4	4	x	0	Poor	Palliative	400	185	Invasion of super mesenteric artery
5	4	x	1	Well	Palliative	600	165	Liver metastasis
6	2	0	0	Poor	Curative	100	165	
7	2	0	0	Moderate	Curative	2300	225	
8	2	1	0	Moderate	Curative	600	205	

the other 7 patients. Major complications were defined as those threatening the life potentially. Neither re-operation was performed nor death of patients occurred during operation. None of the 8 patients was treated in ICU. The curative resection rate of the patients without back pain was higher than that of those with back pain (66.67% *vs* 20%, 2/3 *vs* 1/5). The median survival time was 6 in the palliative resection group and 12 mo in the curative resection group, respectively. The latter group had a longer survival time than the former group, while the former group had a higher 1-year survival rate than the latter group (66.7% *vs* 0%).

DISCUSSION

The prognosis of pancreatic body and tail cancer is poor even after surgical resection[1-10]. Due to the absence of painless obstructive jaundice and earlier symptoms, most patients with pancreatic body and tail cancer are found in an advanced stage, which results in a low resection rate[1-10]. The 5-year survival rate of pancreatic body and tail cancer patients after surgical resection ranges from 0% to 25%, and the median survival time is 10 to 15.9 mo[4-10]. In our study, no NCAPBT patient survived longer than 5 years, the median survival time of the patients after curative resection was comparable with that of other reports [5-10], but the median survival time of the patients after palliative resection was much shorter than that of those after curative resection (Table 2). The portal vein and/or superior mesenteric vein resection combined with pancreatectomy can be successfully performed[9,10], preoperative CT scan or other image analysis should be emphasized on the relationship between the tumor and its major adjacent vessels, such as superior mesenteric artery, abdominal aorta and celiac trunk, because they help surgeons to more precisely judge the resectability of the tumor before operation.

Bathe *et al*[11] reported that old NCAPBT patients have a higher ratio of major postoperative complications and a significantly shorter survival time, and need to receive radical resection. An interesting finding in our study is that three old patients (≥ 70 years of age) had no major postoperative complications, however, 1 of 5 patients (< 70 years of age) had major postoperative complications. No patient received re-operation. Furthermore, 2 patients in the older patient group (≥ 70 years of age) after curative resection had a longer survival time than those after palliative resection, suggesting that surgical resection of NCAPBT in old patients might not result in more major postoperative complications than in younger patients.

Pancreatic cancer patients with back pain have a low resection rate and poor prognosis[12]. In our study, the patients without back pain trended to have more chances of curative resection, however, the longest survivors were those with back pain, indicating that back pain should not be considered a contraindication, though the patients with back pain had less chance of curative resection.

Metastasis occurs in most patients with adenocarcinoma of the body and tail of the pancreas[3,10,13,14]. In our study, one patient with liver metastasis had a comparable

survival time with the other 4 after palliative resection, suggesting that the prognosis of patients with liver metastasis after palliative resection of NCAPBT is not poorer than that of patients without distal metastasis.

Sboup *et al*[10] reported that the survival rate is correlated with histological differentiation. An interesting finding in our study is that three patients with poorly- or moderately-differentiated tumor in the curative resection group had a longer survival time than the other three patients with well-differentiated tumor in the palliative resection group, indicating that though histological differentiation is related with survival, curative resection of NCAPBT is a more important prognostic factor.

Segmental pancreatectomy is mainly performed for benign or low malignant tumor in the pancreatic body and leads to more postoperative complications than distal pancreatectomy[15]. In our study, one patient after palliative segmental pancreatectomy had no major complications and was discharged ten days after the operation. The survival time was a little longer than that of the other 4 patients after palliative resection, suggesting that segmental pancreatectomy is a feasible surgery and can preserve more pancreatic tissues.

Sboup *et al*[10] reported that if extended resection is considered necessary for curative resection of the tumor, the resection is justified in patients with NCAPBT. Fabre *et al*[4] found that only patients with tumor not more than 4 cm in diameter, but without lymph involvement and metastasis have a significant longer survival time after resection. In our study, the patients in the curative resection group had a longer survival time than those in the palliative resection group. The median survival time of the latter group was only half of the former group. No patients survived more than 1 year in the palliative resection group. The data showed that curative resections of NCAPBT should be recommended. Furthermore, in the curative resection group, one patient with a tumor over 4 cm in diameter and lymph involvement, survived only 8 mo which was a little longer than the median survival time of those in the palliative resection group, while survival time of the two patients with tumor not more than 4 cm in diameter but without lymph involvement and metastasis was not less than 1 year, indicating that the theory of Fabre *et al*[4] seems to fit for the Chinese patients.

In conclusion, the overall survival of Chinese NCAPBT patients is dismal. Chinese NCAPBT patients after curative resection may have a longer survival time. Chinese NCAPBT patients with back pain, trend to have a lower curative resection rate, but back pain should not be considered a contraindication for curative NCAPBT resection.

REFERENCES

1 **Dalton RR**, Sarr MG, van Heerden JA, Colby TV. Carcinoma of the body and tail of the pancreas: is curative resection justified? *Surgery* 1992; **111**: 489-494

2 **Nordback IH**, Hruban RH, Boitnott JK, Pitt HA, Cameron JL. Carcinoma of the body and tail of the pancreas. *Am J Surg* 1992; **164**: 26-31

3 **Johnson CD**, Schwall G, Flechtenmacher J, Trede M. Resection for adenocarcinoma of the body and tail of the pancreas. *Br J Surg* 1993; **80**: 1177-1179

4 **Fabre JM**, Houry S, Manderscheid JC, Huguier M, Baumel H. Surgery for left-sided pancreatic cancer. *Br J Surg* 1996; **83**: 1065-1070

5 **Brennan MF**, Moccia RD, Klimstra D. Management of adenocarcinoma of the body and tail of the pancreas. *Ann Surg* 1996; **223**: 506-511; discussion 511-512

6 **Nakao A**, Harada A, Nonami T, Kaneko T, Nomoto S, Koyama H, Kanazumi N, Nakashima N, Takagi H. Lymph node metastasis in carcinoma of the body and tail of the pancreas. *Br J Surg* 1997; **84**: 1090-1092

7 **Sperti C**, Pasquali C, Pedrazzoli S. Ductal adenocarcinoma of the body and tail of the pancreas. *J Am Coll Surg* 1997; **185**: 255-259

8 **Kayahara M**, Nagakawa T, Ueno K, Ohta T, Kitagawa H, Arakawa H, Yagi H, Tajima H, Miwa K. Distal pancreatectomy-does it have a role for pancreatic body and tail cancer. *Hepatogastroenterology* 1998; **45**: 827-832

9 **Burcharth F**, Trillingsgaard J, Olsen SD, Moesgaard F, Federspiel B, Struckmann JR. Resection of cancer of the body and tail of the pancreas. *Hepatogastroenterology* 2003; **50**: 563-566

10 **Shoup M**, Conlon KC, Klimstra D, Brennan MF. Is extended resection for adenocarcinoma of the body or tail of the pancreas justified? *J Gastrointest Surg* 2003; **7**: 946-952; discussion 952

11 **Bathe OF**, Caldera H, Hamilton KL, Franceschi D, Sleeman D, Livingstone AS, Levi JU. Diminished benefit from resection of cancer of the head of the pancreas in patients of advanced age. *J Surg Oncol* 2001; **77**: 115-122

12 **Ridder GJ**, Klempnauer J. Back pain in patients with ductal pancreatic cancer. Its impact on resectability and prognosis after resection. *Scand J Gastroenterol* 1995; **30**: 1216-1220

13 **Sener SF**, Fremgen A, Menck HR, Winchester DP. Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985-1995, using the National Cancer Database. *J Am Coll Surg* 1999; **189**: 1-7

14 **Jimenez RE**, Warshaw AL, Rattner DW, Willett CG, McGrath D, Fernandez-del Castillo C. Impact of laparoscopic staging in the treatment of pancreatic cancer. *Arch Surg* 2000; **135**: 409-414; discussion 414-415

15 **Shibata S**, Sato T, Andoh H, Yasui O, Yoshioka M, Kurokawa T, Watanabe G, Ise N, Kotanagi H, Asanuma Y, Koyama K. Outcomes and indications of segmental pancreatectomy. Comparison with distal pancreatectomy. *Dig Surg* 2004; **21:** 48-53

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CASE REPORT

Hepatic intra-arterial infusion of yttrium-90 microspheres in the treatment of recurrent hepatocellular carcinoma after liver transplantation: A case report

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Abstract

Hepatocellular carcinoma (HCC) recurs with a reported frequency of 12%-18% after liver transplantation. Recurrence is associated with a mortality rate exceeding 75%. Approximately one-third of recurrences develop in the transplanted liver and are therefore amenable to local therapy. A variety of treatment modalities have been reported including resection, transarterial chemoembolization (TACE), radiofrequency ablation (RFA), ethanol ablation, cryoablation, and external beam irradiation. Goals of treatment are tumor control and the minimization of toxic effect to functional parenchyma. Efficacy of treatment is mitigated by the need for ongoing immunosuppression. Yttrium-90 microspheres have been used as a treatment modality both for primary HCC and for pre-transplant management of HCC with promising results.

Twenty-two months after liver transplantation for hepatitis C cirrhosis complicated by HCC, a 42-year old man developed recurrence of HCC in his transplant allograft. Treatment of multiple right lobe lesions with anatomic resection and adjuvant chemotherapy was unsuccessful. Multifocal recurrence in the remaining liver allograft was treated with hepatic intra-arterial infusion of yttrium-90 microspheres (SIR-Spheres, Sirtex Medical Inc., Lake Forest, IL, USA). Efficacy was demonstrated by tumor necrosis on imaging and a decrease in alpha-fetoprotein (AFP) level. There were no adverse consequences of initial treatment.

Key words: Recurrent hepatocellular carcinoma; Yttrium-90 microspheres; Liver transplantation

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INTRODUCTION

Strict criteria developed to select patients with hepatocellular carcinoma (HCC) for liver transplantation have increased the survival and decreased the recurrence of tumor after transplantation[1]. However, tumor recurrence and subsequent mortality continue to be observed. Tumor recurrence is reported to occur with a frequency of 12%-18%[2-4]. Seventy percent of recurrences are observed within the first year after transplantation[3]. Unfortunately, recurrence-related mortality is high and exceeds 75%[2]. Extra-hepatic recurrence is encountered most commonly, with an estimated incidence of 67%[2]. Recurrence isolated to the transplant liver is relatively infrequent, yet, it represents an opportunity for targeted treatment. Local therapies include resection, trans-arterial chemoembolization (TACE), radiofrequency ablation (RFA), ethanol ablation, cryoablation, and external beam irradiation. Treatment efficacy varies according to tumor characteristics such as location, size, and number. Efficacy also varies with the degree of underlying hepatic dysfunction. Hepatic arterial infusion of microspheres impregnated with the beta-radiation emitter yttrium-90 has been successfully employed in the palliative treatment of unresectable HCC and most recently to down-stage HCC to allow liver transplantation[5]. Results for the treatment of primary HCC reveal a 38%-65% partial response rate, and a median survival of 23 mo which is 2.6-4.7 times the length of median survival seen in historic controls[6].

The following case report describes a 42-year old man who underwent liver transplantation for hepatitis C

cirrhosis with stage II HCC and 22 mo later developed recurrent HCC isolated to the liver allograft. When conventional treatment modalities failed, hepatic arterial infusion of yttrium-90 beads (SIR-Spheres, Sirtex Medical Inc., Lake Forest, IL, USA) was safely employed to treat the HCC recurrence in the liver allograft.

CASE REPORT

This hispanic man was diagnosed at 38 years of age with end-stage liver dysfunction secondary to hepatitis C infection (genotype 1a) and listed with UNOS for transplantation in 2001 (UNOS status 3, MELD score 12). Nine months after being listed, serial CT examination of the liver suggested a new 1.5 cm lesion in segment IV. Follow-up imaging five months later showed the lesion to have grown in size to 3 cm consistent with HCC (stage Ⅱ, T2NxMx). AFP level at this time was 38.7 μg/L. He underwent percutaneous RFA of the lesion while awaiting transplantation with a slight decrease in the AFP level to 32.9 μg/L and no residual tumor on CT after treatment. He received the standard MELD exception score at the time of 29 for his stage Ⅱ HCC. A deceased donor liver transplant became available 5 mo after receiving his exception score (3.5 mo after RFA and 23 mo after being originally listed for transplant). The donor was a 52-year old CMV-positive man. Standard caval interposition technique without veno-venous bypass was used and the total graft ischemic time was 7 h and 40 min. Induction immunotherapy consisted of 20 mg of basiliximab, 300 mg of azathioprine and 1 g of methylprednisolone. Contrary to the post-RFA CT scan from 10 wk earlier, pathologic evaluation of the hepatic explant was notable for the presence of three foci of HCC in the right lobe of the liver. The lesions measured 2.8 cm, 2.7 cm and 1.5 cm each, in maximum dimension. The largest was 60% necrotic as a result of RFA treatment. Each tumor was moderately or well differentiated and demonstrated no angiolymphatic invasion.

Following transplantation, steroid doses were tapered to 15 mg/d by postoperative day seven. Azathioprine was discontinued on postoperative day seven and maintenance immunosuppression with tacrolimus was begun. His first allograft biopsy three weeks after transplant revealed only mild ischemia-reperfusion changes and no evidence of rejection or recurrent HCV infection. The tacrolimus dose was adjusted to achieve a trough of 10-15 μg/L for the first three months after transplant. After three months, transaminases were elevated and a liver biopsy done at this time revealed recurrent HCV with grade 1 (0-4) inflammation and steatosis without fibrosis (stage 0, 0-4). Prednisone dose was tapered and subsequently discontinued. Tacrolimus doses were further decreased to achieve a target trough level of 6-10 μg/L. A one-year liver biopsy, however, demonstrated progression to fibrosis (stage 2, 0-4). Transaminases remained mildly elevated and new hyperbilirubinemia (total bilirubin 29.1 μmol/L) was also noted. Quantitative HCV RNA testing revealed high-level viremia with viral tiers of 5.8 GU/L. HCV treatment with pegylated interferon and ribavirin was begun. After a 15 wk course of interferon-based anti-viral therapy, he had only a transient decrease in transaminases and bilirubin with persistently high HCV RNA levels. HCV treatment was discontinued.

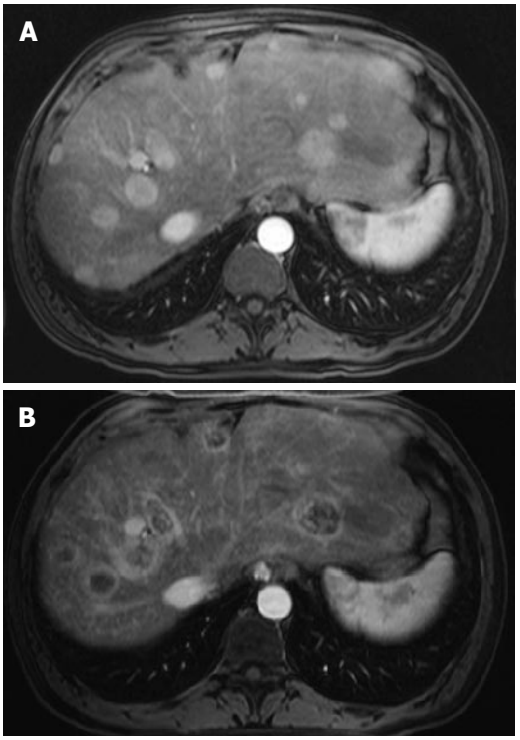

Figure 1 Pre-treatment magnetic resonance imaging of the liver demonstrating multifocal recurrent tumor within liver allograft (**A**) and paired post-treatment image showing central necrosis of lesions after treatment with yttrium-90 microspheres (**B**).

HCC surveillance with serial imaging and AFP levels remained negative for the first 18 mo following transplantation. An elevated AFP level of 16 μg/L was noted at 22 mo post transplant. A CT scan of the liver revealed a 3 cm nodule in segment VI not seen on the imaging five months earlier. Recurrent HCC was confirmed by needle biopsy, bone scan and chest CT were negative for distant metastatic disease. The patient was offered a segmental resection of the tumor and underwent laparotomy. No extra-hepatic tumor was identified. Intraoperative ultrasound revealed two additional 1 cm lesions in segments Ⅲ and Ⅳ. These and the initial segment Ⅵ lesion were segmentally resected. The patient's post-operative course was uncomplicated. Post-operative AFP level decreased to 7 μg/L. One month after surgery, maintenance immunotherapy was switched from tacrolimus monotherapy to sirolimus monotherapy with target trough levels of 5-8 μg/L and adjuvant chemotherapy with systemic doxorubicin (200 mg/m^2 over 20 wk) was begun. Chemotherapy was complicated by the development of diabetes mellitus and anemia. AFP level increased to 820 μg/L despite chemotherapy. A CT scan done 3 mo into his course of doxorubicin showed vague hyper-enhancement in segment Ⅷ and magnetic resonance imaging (MRI) scan of the liver obtained at the completion of treatment revealed uniformly enhancing, multifocal nodules consistent with HCC in the remaining liver allograft with the largest nodule measuring 3.8 cm (Figure 1A).

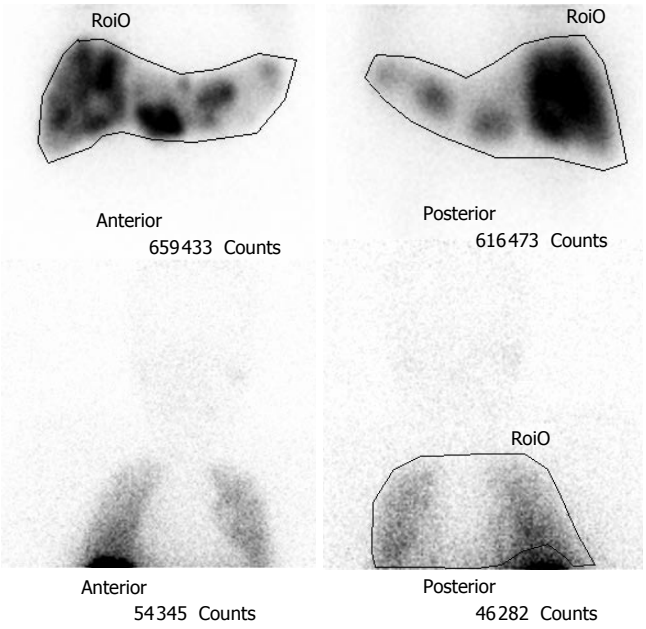

Figure 2 Pre-treatment screening by injection of macroaggregated albumin to determine shunt fraction to lungs.

After presentation at a multidisciplinary tumor board it was decided to proceed with a trial of hepatic intra-arterial radiotherapy with yttrium-90 microspheres (SIR-Spheres). Pre-procedure evaluation with hepatic arteriography was notable for the absence of collateral vessels to the allograft, given that the liver was supplied by the donor vessels and the native gastroduodenal artery was ligated at the time of transplantation. Injection of macroaggregated albumin to determine the degree of hepato-pulmonary shunting revealed a radioactive shunt fraction of 7.3% (Figure 2). CT volumetry of the affected liver allowed calculation of a targeted tumor volume of 120.6 cm^3. He underwent yttrium-90 intra-hepatic arterial injection 32 mo after liver transplantation. The patient received a dose of 1.5 GBq of isotope. The procedure was uncomplicated and he was discharged to home six hours after the procedure was completed. The patient experienced only minimal symptoms related to the treatment, including mild right upper quadrant abdominal pain and intermittent nausea. Follow-up MRI of the liver was done two months post-treatment. Pre- and post treatment MRI images (Figure 1A and B) demonstrated loss of uniform arterial enhancement and development of peripheral enhancement consistent with tumor necrosis and treatment response. The number of lesions remained stable. Post-procedure AFP levels decreased to 20 μg/L at 2 mo following the procedure. Liver function, both clinical and biochemical, was unaffected by the treatment.

DISCUSSION

Following liver transplantation, recurrence of HCC that is isolated to the liver occurs with a frequency of around 3%-5% with the current liver transplant candidate selection criteria[2-4]. Most recurrences occur within the first two years of transplantation and the two-year mortality after recurrence is high, approaching 75%[4]. Recurrent HCC may be heralded by impairment in graft function and elevation of AFP levels. Treatment measures usually include reduction of immunosuppression and local tumor therapy. Hepatic recurrence is multifocal in 60% of cases and tumor grade is T3 or greater in up to 90% of cases[3]. As a result, impaired hepatic function along with multifocal disease often limits the potential for surgical resection. Local ablative techniques such as RFA, cryoablation or percutaneous ethanol ablation require repeated administrations and also have limited efficacy when there is extensive or diffuse tumor burden. TACE has evolved as a useful tool for treatment of HCC recurrence because it can be utilized for treatment of bilateral, multifocal lesions with efficacy and limited repeated administration[6]. However, treatment requires infusion of chemotherapy and embolization of the selected hepatic artery. Systemic and local toxicity as a result of chemotherapy and hepatic ischemia is not rare[6].

This is the first known report of hepatic intra-arterial brachytherapy with yttrium 90 microsphere (SIR-Spheres) for the treatment of HCC in a liver transplant recipient. Intrahepatic yttrium 90 microsphere (SIR-Spheres) has been used for the treatment of unresectable HCC and metastatic hepatic tumors[7,8]. Yttrium-90 isotopes incorporated into a resin sphere slightly larger than the size of the capillary in the liver arterial system are delivered intra-arterially. Yttrium 90 emits beta-radiation and the permanently implanted spheres deliver a therapeutic dose radiation with an effective mean range of about 2.5 mm. The half-life of the isotope is 64 h with 95% of the dose given in the first 11 d. Higher doses of radiation are able to be delivered selectively than with external beam radiation therapy (EBRT). In addition, large segments of liver are not rendered ischemic because embolization is not a feature of the therapy. Therefore, there is less toxicity to functioning parenchyma than seen with EBRT or TACE.

Therapy is typically administered in one cycle, with a range of one-to-three cycles. Suitable candidates for treatment include patients with unilobar or bilobar disease and preserved liver function with bilirubin < 34.2 μmol/L[7]. Contrast enhanced CT imaging of the liver serves to determine tumor volume and allow calculation of radiation dose requirement. In order to minimize treatment-related toxicity, hepatic arteriography is necessary to determine arterial anatomy and administer 99 mTc-macro-aggregated albumin to calculate the shunt fraction delivered to the lungs. In most patients, arteries that arise from the hepatic arteries and supply the stomach or intestines must be embolized to prevent radiation necrosis. If the hepatic arterial supply cannot be isolated, then the patient is generally considered not to be a candidate for therapy. Because of the isolated arterial supply of the liver allograft in our patient, this was not an issue. Pulmonary shunt fraction greater than 15% on 99 mTc-macro-aggregated albumin scan predisposes to radiation pneumonitis and precludes safe treatment with yttrium 90.

The median radiation dose for a treatment is 134 Gy which is delivered in a range of 50-150 Gy[7]. The dose of yttrium 90 equates to 5-10 GBq or 2-4 million microspheres. Treatment is typically provided on an outpatient basis and does not require patient isolation

because yttrium 90 is a beta emitter. Local response to treatment is demonstrated by decrease in size and tumor vascularity. Thirty-eight percent of patients experience partial response demonstrated by a decrease in tumor size[7]. In 65% of patients, response is demonstrated by substantial decrease in tumor vascularity[7]. Monitoring of tumor markers such as AFP may also be of benefit in determining response to therapy[8]. Impact on survival varies according to the severity of underlying hepatic disease but the median survival time after yttrium 90 microsphere (Theraspheres, MDS Nordion, Ottowa, Canada) treatment was 23 mo in one study of 65 patients with unresectable HCC[7]. This represents an increase in survival of 2.6-4.7 times that of historic control. One multicenter review of treated patients reported that death resulting from therapy-induced liver failure is responsible for half of the treatment-related deaths and occurs in 5% of all patients[9]. All of these liver failure deaths attributed to the yttrium 90 therapy are occurred in patients felt to be at high risk for the procedure. When compared to TACE, quality of life measures is higher at 12 mo[10]. Toxicities of yttrium-90 include worsening ascites, lower extremity edema, nausea, vomiting, fever, abdominal pain, hyperbilirubinemia and lymphopenia[7,8]. Mild adverse effects include nausea, vomiting fatigue, abdominal pain, fever[7,8]. Serious adverse effects include tumor lysis syndrome, transient elevations in liver enzymes, gastritis, gastric or duodenal perforation, cholecystitis, and radiation induced pneumonitis[7,8].

A recent multicenter review of low risk patients treated with yttrium 90 microspheres reported that liver toxicities including elevation of bilirubin and serum transaminase levels and ascites occur after treatment in 42% of patients, fifty percent of these adverse events are thought to be related to treatment, and toxicities are resolved in 78% of patients on short term follow-up[11]. Development of toxicity is associated with pretreatment bilirubin > 17.2 μmol/L and radiation dose > 137 Gy. There are no occurrences of radiation-induced liver disease or death related to treatment[11]. Another multicenter review on the use of intrahepatic yttrium 90 microspheres has reported a three-month post-treatment mortality of 18%. Ten percent of deaths are thought to be related to treatment and occur in association with at least one or more of the following risk factors: presence of infiltrative tumor, tumor bulk > 50%, albumin < 30 g/L, bilirubin ⩾ 34.2 μmol/L, AST or ALT > five times the upper limit of normal, and a lung radiation dose of > 30 Gy[9]. The results of these and other reports suggest that treatment in carefully selected patients is safe and efficacious.

Hepatic intra-arterial yttrium-90 microsphere treatment was safely and effectively used in this patient with recurrent multifocal HCC after liver transplantation. Clearly, further prospective investigation is needed to determine whether this therapy can be applied with similar safety and efficacy to other patients with recurrent HCC after liver transplantation. The availability of this treatment modality in the post-transplant setting may help to prolong patient and graft survival in those recipients unfortunate enough to develop recurrent HCC who are not amenable to other treatment options.

REFERENCES

1 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699

2 **Island ER**, Pomposelli J, Pomfret EA, Gordon FD, Lewis WD, Jenkins RL. Twenty-year experience with liver transplantation for hepatocellular carcinoma. *Arch Surg* 2005; **140**: 353-358

3 **Vivarelli M**, Bellusci R, Cucchetti A, Cavrini G, De Ruvo N, Aden AA, La Barba G, Brillanti S, Cavallari A. Low recurrence rate of hepatocellular carcinoma after liver transplantation: better patient selection or lower immunosuppression? *Transplantation* 2002; **74**: 1746-1751

4 **Roayaie S**, Schwartz JD, Sung MW, Emre SH, Miller CM, Gondolesi GE, Krieger NR, Schwartz ME. Recurrence of hepatocellular carcinoma after liver transplant: patterns and prognosis. *Liver Transpl* 2004; **10**: 534-540

5 **Kulik LM**, Mulcahy MF, Hunter RD, Nemcek AA Jr, Abecassis MM, Salem R. Use of yttrium-90 microspheres (TheraSphere) in a patient with unresectable hepatocellular carcinoma leading to liver transplantation: a case report. *Liver Transpl* 2005; **11**: 1127-1131

6 **Chan AO**, Yuen MF, Hui CK, Tso WK, Lai CL. A prospective study regarding the complications of transcatheter intraarterial lipiodol chemoembolization in patients with hepatocellular carcinoma. *Cancer* 2002; **94**: 1747-1752

7 **Carr BI**. Hepatic arterial 90Yttrium glass microspheres (Therasphere) for unresectable hepatocellular carcinoma: interim safety and survival data on 65 patients. *Liver Transpl* 2004; **10**: S107-S110

8 **Salem R**, Thurston KG, Carr BI, Goin JE, Geschwind JF. Yttrium-90 microspheres: radiation therapy for unresectable liver cancer. *J Vasc Interv Radiol* 2002; **13**: S223-S229

9 **Goin JE**, Salem R, Carr BI, Dancey JE, Soulen MC, Geschwind JF, Goin K, Van Buskirk M, Thurston K. Treatment of unresectable hepatocellular carcinoma with intrahepatic yttrium 90 microspheres: a risk-stratification analysis. *J Vasc Interv Radiol* 2005; **16**: 195-203

10 **Steel J**, Baum A, Carr B. Quality of life in patients diagnosed with primary hepatocellular carcinoma: hepatic arterial infusion of Cisplatin versus 90-Yttrium microspheres (Therasphere). *Psychooncology* 2004; **13**: 73-79

11 **Goin JE**, Salem R, Carr BI, Dancey JE, Soulen MC, Geschwind JF, Goin K, Van Buskirk M, Thurston K. Treatment of unresectable hepatocellular carcinoma with intrahepatic yttrium 90 microspheres: factors associated with liver toxicities. *J Vasc Interv Radiol* 2005; **16**: 205-213

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CASE REPORT

Successful management of hepatic artery pseudoaneurysm complicating chronic pancreatitis by stenting

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Abstract

A 41-year old alchoholic male with a history of chronic pancreatitis was admitted for nausea, vomiting and weight loss. Angiogram was performed and demonstrated an aneurysmal sac with a narrow neck originating from the inferior aspect of the distal portion of the proper hepatic artery. The origin of the pseudoaneurysm was covered with a 5 mm × 2.5 cm Viabahn cover stent (Gore). A repeat angiogram showed some leak and a second stent (6 mm × 2.3 cm) was deployed and overlapped with the first stent by 3 mm. Contrast was injected and a repeat angiogram demonstrated complete exclusion of the aneurysm. A repeat computerized axial tomography (CAT) scan of the abdomen after 24 h showed successful stenting. The patient had an uneventful post-operative course.

Key words: Hepatic artery; Aneurysm; Stents

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INTRODUCTION

Hepatic artery pseudoaneurysm (HAP) is a serious complication of acute or chronic surgical injury to the hepatic artery. It is also seen following blunt and penetrating abdominal injury as well as in patients with chronic pancreatitis and after orthotopic liver transplantation[1]. Transcatheter embolization has been considered the treatment of choice but we report a case of HAP successfully treated by stenting of the pseudoaneurysm.

CASE REPORT

A 41-year old male with a history of chronic alchohol consumption and chronic recurrent pancreatitis presented to the emergency department with abdominal pain, nausea and weight loss. Physical examination revealed remarkable ascites. No bruit was present. Serum upper gastrointestinal (GI) endoscopy revealed grade 1 esophageal varices. Abdominal ultrasonography (USG) demonstrated dilated pancreatic ducts. Abdominal MRI/MRA showed a lesion in the porta hepatis measuring 5 cm × 5 cm. Contrast-enhanced CAT scan of the abdomen showed a 6 cm × 6 cm pseudoaneurysm of the proper hepatic artery (Figure 1). Stenting of the pseudoaneurysm was done. The technique could selectively catheterize the proper hepatic artery by using a 5 French RC-1 catheter (Angiodynamic-Queensbury-NY). Angiogram was performed and demonstrated an aneurysmal sac with a narrow neck originating from the inferior aspect of the distal portion of the proper hepatic artery (Figure 2). The origin of the pseudoaneurysm was covered with a 5 mm × 2.5 cm Viabahn cover stent (Gore). A repeat angiogram showed some leak and a second stent (6 mm × 2.3 cm) was deployed and overlapped with the first stent by 3 mm. Contrast was injected and a repeat angiogram demonstrated complete exclusion of the aneurysm (Figure 3). A repeat computerized axial tomography (CAT) scan of the abdomen after 24 h showed successful stenting (Figure 4). The patient had an uneventful post-operative course.

DISCUSSION

Hepatic artery pseudoaneurysms are rare. However, rupture is common and occurs in 76% of patients. The mortality of patients requiring operative intervention is 75%[1]. Psudoaneurysm formation in pancreatitis is thought to occur because of autodigestion of pancreatic enzymes, especially elastase, liberated due to pancreatitis. Although angiography remains the gold standard for detection of pancreatitis, USG and CAT scan are often diagnostic and may provide an early mechanism for identifying patients with pseudoaneurysms occurring consequently to pancreatitis[2]. Otherwise diagnosis is usually made late in the course of disease when severe or even fatal hemorrhage has occurred.Transcatheter embolization is the standard of care.

Literature search revealed one case report of a 60-year old female with HAP as a complication of surgical treatment of Klatskin tumor treated with a

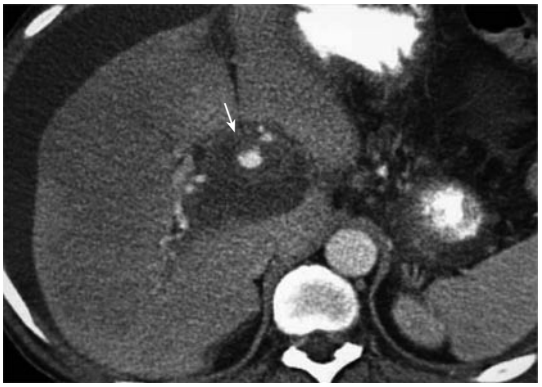

Figure 1 Contrast-enhanced CAT of the abdomen showing a 6 cm × 6 cm pseudoaneurysm of the proper hepatic artery.

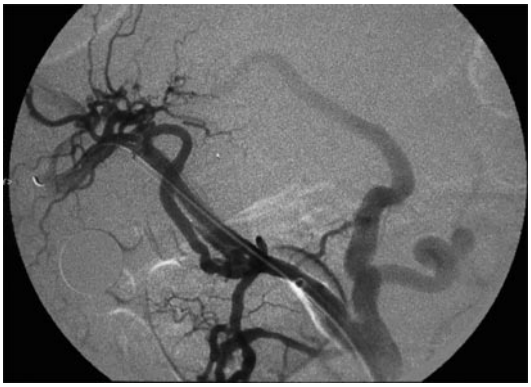

Figure 3 Repeat angiogram demonstrating complete exclusion of the aneurysm.

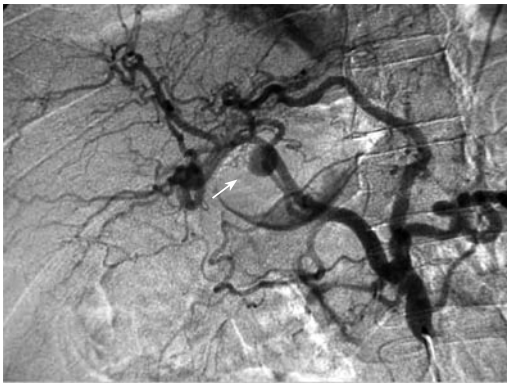

Figure 2 Angiogram showing an aneurysmal sac with a narrow neck originating from the inferior aspect of the distal portion of the proper hepatic artery.

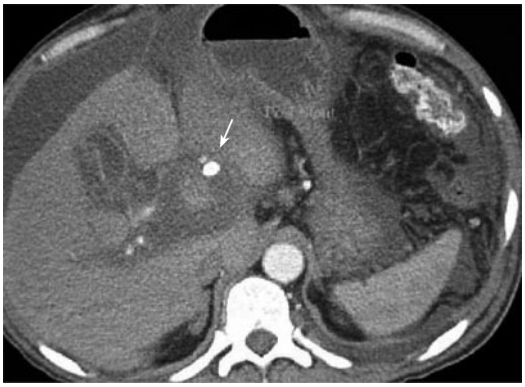

Figure 4 A repeat CAT scan of the abdomen after 24 h showing successful stenting.

coronary stent-graft via transfemoral approach[3]. Two other cases have been reported, one being stenting of HAP secondary to liver transplantation following unsuccessful coil embolization[4] and the other being stenting of HAP secondary to therapeutic management of a choledochol cholangiocarcenoma, following failed arterial embolization[5]. In our case HAP developed as a complication of chronic alchoholic pancreatitis with symptoms of abdominal pain. Early diagnosis with CAT scan followed by stenting resulted in complete occlusion of the pseudoaneurysm.

The long-term data regarding the use of this or other stent-grafts in the visceral vessels are extremely limited, and consequently we cannot recommend stent-grafts as the primary treatment option for visceral artery pseudoaneurysms. However, we expect that, with continued follow-up of cases such as these, further refinements in stent-graft design, greater availability, and increased operator experience, these devices will become an increasingly valuable therapeutic option. Early diagnosis and intervention with stenting, which is a reasonable alternative to embolization, can result in excellent long-term outcome.

REFERENCES

1 **Finley DS**, Hinojosa MW, Paya M, Imagawa DK. Hepatic artery pseudoaneurysm: a report of seven cases and a review of the literature. *Surg Today* 2005; **35**: 543-547

2 **Tessier DJ**, Fowl RJ, Stone WM, McKusick MA, Abbas MA, Sarr MG, Nagorney DM, Cherry KJ, Gloviczki P. Iatrogenic hepatic artery pseudoaneurysms: an uncommon complication after hepatic, biliary, and pancreatic procedures. *Ann Vasc Surg* 2003; **17**: 663-669

3 **Venturini M**, Angeli E, Salvioni M, De Cobelli F, Trentin C, Carlucci M, Staudacher C, Del Maschio A. Hemorrhage from a right hepatic artery pseudoaneurysm: endovascular treatment with a coronary stent-graft. *J Endovasc Ther* 2002; **9**: 221-224

4 **Maleux G**, Pirenne J, Aerts R, Nevens F. Case report: hepatic artery pseudoaneurysm after liver transplantation: definitive treatment with a stent-graft after failed coil embolisation. *Br J Radiol* 2005; **78**: 453-456

5 **Paci E**, Antico E, Candelari R, Alborino S, Marmorale C, Landi E. Pseudoaneurysm of the common hepatic artery: treatment with a stent-graft. *Cardiovasc Intervent Radiol* 2000; **23**: 472-474

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CASE REPORT

Obstructive jaundice due to hepatobiliary cystadenoma or cystadenocarcinoma

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Abstract

Hepatobiliary cystadenomas (HBC) and cystadenocarcinomas are rare cystic lesions. Most patients with these lesions are asymptomatic, but presentation with obstructive jaundice may occur. The first patient presented with intermittent colicky pain and recurrent obstructive jaundice. Imaging studies revealed a polypoid lesion in the left hepatic duct. The second patient had recurrent jaundice and cholangitis. Endoscopic retrograde cholangiopancreatography (ERCP) showed a cystic lesion at the confluence of the hepatic duct. In the third patient with intermittent jaundice and cholangitis, cholangioscopy revealed a papillomatous structure protruding into the left bile duct system. In the fourth patient with obstructive jaundice, CT-scan showed slight dilatation of the intrahepatic bile ducts and dilatation of the common bile duct of 3 cm. ERCP showed filling of a cystic lesion. All patients underwent partial liver resection, revealing HBC in the specimen. In the fifth patient presenting with obstructive jaundice, ultrasound examination showed a hyperechogenic cystic lesion centrally in the liver. The resection specimen revealed a hepatobiliary cystadenocarcinoma. HBC and cystadenocarcinoma may give rise to obstructive jaundice. Evaluation with cross-sectional imaging techniques is useful. ERCP is a useful tool to differentiate extraductal from intraductal obstruction.

Key words: Liver; Hepatobiliary cystadenoma; Cystadenocarcinoma; Obstructive jaundice; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Hepatobiliary cystadenomas (HBC) are rare neoplasms of the liver or extrahepatic bile ducts, accounting for less than 5% of all the cystdenomas found in the liver. These lesions are mainly seen in middle-aged females[1,2] and can show malignant degeneration to hepatobiliary cystadenocarcinoma. Most of these patients are asymptomatic, because these lesions usually are incidental findings on abdominal diagnostic imaging for evaluation of other complaints. If there are presenting symptoms, these are right upper quadrant pain or discomfort. Obstructive jaundice only rarely occurs as a presenting symptom[3]. We describe here the diagnostic evaluation, surgical management and pathological characteristics of four patients with HBC and one patient with hepatobiliary cystadenocarcinoma who presented with obstructive jaundice.

CASE REPORT

Case 1

A 50-year old woman underwent laparoscopic cholecystectomy because of symptomatic cholecystolithiasis 3 years prior to presentation. She was admitted for intermittent colicky pain and recurrent biliary obstruction. Physical examination showed no abnormalities. Routine liver function tests revealed elevated plasma levels of alkaline phospatase (AP) of 118 U/L (normal range, 40-120 U/L) and gamma-glutamyltransferase (GGT) of 260 U/L (normal, < 40 U/L). Serum bilirubin level was normal. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated a polypoid lesion inside the left hepatic duct. Helical computed tomography (CT) of the liver revealed a cystic lesion measuring 10 cm in diameter located in the left lobe of the liver with protrusion into the left hepatic duct. Calcifications were present in the wall of the lesion and septations were visible inside the cyst (Figure 1A). ERCP showed the image of an extrinsic mass proximal to the polypoid lesion, creating a compression on the intrahepatic ducts of the left biliary system (Figure 1B). These findings suggested that the episodes of colicky pain and cholestasis were caused by a cystic lesion with a polypoid protrusion into the left bile duct, giving rise to intermittent obstruction. Distinction between hepatobiliary cystadenoma and cystadenocarcinoma could not be made on the basis of imaging studies. The patient underwent left hemihepatectomy and

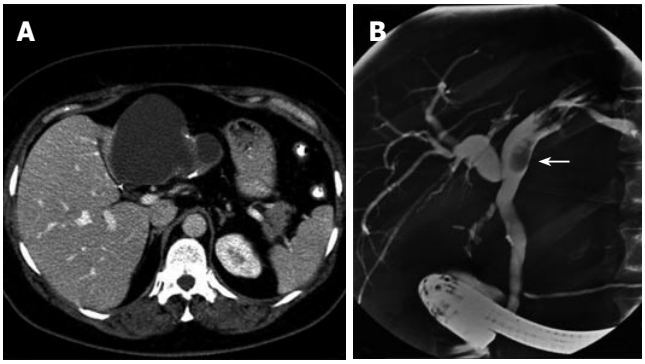

Figure 1 Abdominal CT-scan showing a large cystic mass in the left liver lobe with internal septations and calcifications in the cyst wall (**A**) and ERCP showing a polypoid lesion in the left hepatic duct (arrow) in case 1 (**B**).

the histopathological diagnosis of hepatobiliary mucinous cystadenoma was made. The postoperative course was uneventful and the complaints resolved after operation. The patient died 56 mo after operation because of other reasons not related to the initial disease.

Case 2

A 46-year old woman underwent cholecystectomy with common bile duct exploration because of gallstones 6 years prior to presentation. During cholecystectomy, fenestration of a presumed simple intraluminal cyst at the confluence of the hepatic duct was performed. Microscopic examination of the cyst wall showed biliary epithelium without mesenchymal stroma. She had recurrent jaundice and cholangitis. Initial investigations revealed elevated levels of serum bilirubin level of 79 μmol/L (normal, < 17 μmol/L), AP of 311 U/L (normal range, 40-120 U/L), GGT of 448 U/L (normal, < 40 U/L), AST of 96 U/L and ALT of 142 U/L (normal, < 45 U/L). ERCP showed filling of a cystic lesion at the confluence of the hepatic duct. Brush cytology revealed no malignancy. A plastic stent was placed for decompression of the biliary system. Abdominal ultrasonography (US) and CT-scan revealed multiple cystic lesions involving segment 4 of the liver with dilatation of the left central and peripheral intrahepatic bile ducts and the right central intrahepatic bile ducts. At laparotomy, multiple cysts in segment 4 of the liver were found with a dominant cystic lesion of 2.5 cm located in the area of hepatic hilum. Hilar resection in combination with left hemihepatectomy was performed. Microscopically, the cyst wall was composed of cylindrical epithelium. The features of the subepithelial stroma were typical for the diagnosis hepatobiliary mucinous cystadenoma. The complaints resolved after operation. The patient was still alive 103 mo after operation.

Case 3

A 40-year old woman underwent laparoscopic cholecystectomy for symptomatic cholecystolithiasis 4 mo ago. She had intermittent complaints of jaundice and cholangitis. Liver function tests showed slightly elevated AP levels of 103 U/L and GGT level of 44 U/L. The serum bilirubin and alanine transaminase levels were normal. ERCP revealed a filling defect in the left hepatic duct (Figure 2A).

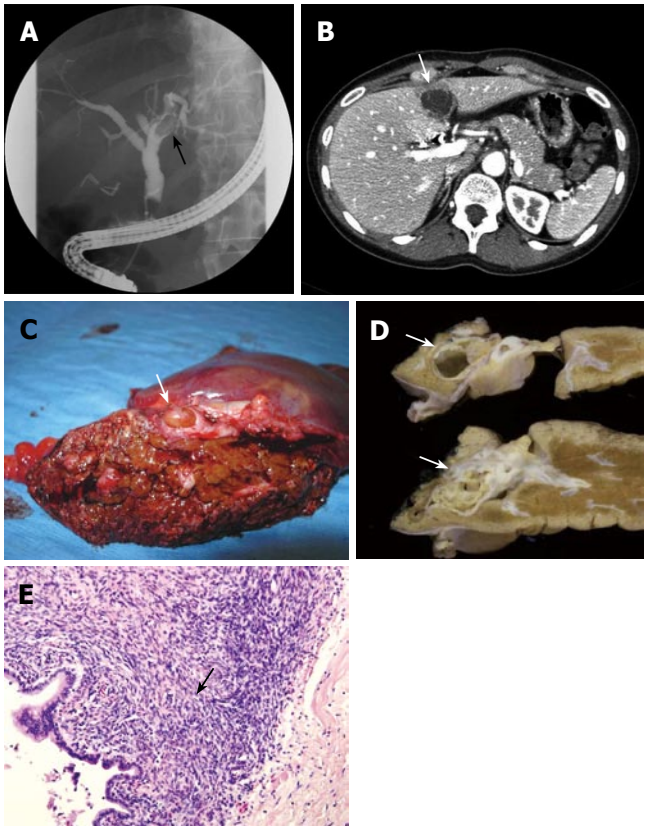

Figure 2 ERCP showing a filling defect due to an intraluminal lesion (arrow) in the left hepatic duct (**A**), CT-scan showing a cystic lesion with internal septations measuring 3.6 cm located in segment 4 (arrow) (**B**), specimen after left hemihepatectomy showing macroscopic features of a large lesion (arrow) inside the left bile duct filling up the entire lumen (**C**), macroscopical cut sections of a multicystic lesion (arrows) encapsulated by a thick fibrous capsule arising from the left hepatic duct (**D**), microscopical features showing columnar mucinous epithelium with underlying dense-cellular stroma resembling ovarian stroma (arrow) (HE X 200) (**E**) in case 3.

A stent was placed for decompression of the biliary system. The second ERCP at our hospital suggested that the filling defect was caused by an intraductal tissue mass. Subsequent cholangioscopy showed a papillomatous structure protruding into the left bile duct system from the bile duct wall. CT scan showed a cystic lesion with septations located in segment 4 of the liver. It was unclear if there was a connection between the lesion and the left hepatic duct. The lesion was 3.6 cm in diameter (Figure 2B). The patient underwent a left hemihepatectomy. Macroscopically, a tumour with smooth surface was seen protruding inside the bile duct and filling up the entire lumen (Figure 2C). Cut sections revealed a multicystic lesion encapsulated by a thick fibrous capsule arising from the left hepatic duct (Figure 2D). The cystic lesion was surrounded by stroma containing spindle-shaped cells, resembling ovarian stroma (Figure 2E). The diagnosis of hepatobiliary mucinous cystadenoma was made. No signs of malignancy were seen. The patient remained alive 19 mo after operation.

Case 4

A 43-year old woman with an unremarkable previous medical history presented with progressive obstructive jaundice three weeks ago. Abdominal US showed dilatation of in-

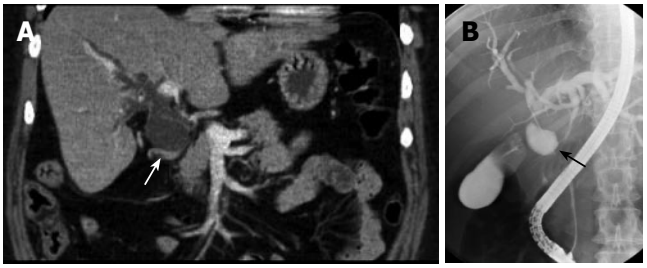

Figure 3 Abdominal coronal CT-scan showing dilated intrahepatic bile ducts and common bile duct (arrow) (**A**), ERCP showing filling of a cystic lesion (arrow) connected to the common bile duct, initially diagnosed as a duplicate gallbladder or choledochal cyst (**B**) in case 4.

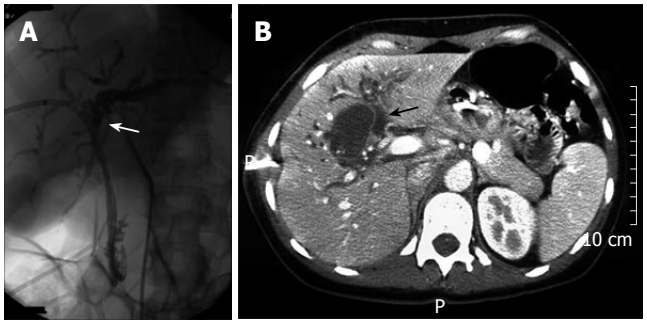

Figure 4 Percutaneous transhepatic drainage (PTD) showing complete obstruction at level of the proximal bile duct (**A**), abdominal CT showing a cystic lesion with irregularly thickened wall in conjunction with dilatated intrahepatic bile ducts (arrow) (**B**) in case 5.

tra- and extra-hepatic bile ducts and a collapsed gallbladder without a clear cause. ERCP showed a normal pancreatic duct but the common bile duct could not be visualized. CT showed slight dilatation of the intrahepatic bile ducts and dilatation of part of the common bile duct with a diameter of 3 cm (Figure 3A). Subsequent MRCP showed the same images as obtained with CT. ERCP showed filling of a cystic lesion connected to the common bile duct (Figure 3B). A diagnosis of a duplicate gallbladder or choledochal cyst at the level of the common hepatic duct was considered. A cystic lesion was detected during laparotomy and a decision was made to resect the common bile duct and the gallbladder. Continuity of the bile duct was restored using a hepaticojejunostomy. Histopathological examination of the resection specimen revealed a multilocular hepatobiliary cystadenoma with necrotizing inflammation. The patient was still alive 46 mo after operation.

Case 5

A 39-year old woman presented with obstructive jaundice, nausea and weight loss which began three months before presentation. Liver function tests showed elevated bilirubin levels of 128 μmol/L, AP of 1053 U/L, GGT of 500 U/L, AST of 172 U/L and ALT of 142 U/L. The alpha-fetoprotein level was normal. Because of failure of ERCP, percutaneous transhepatic drainage (PTD) was preformed for biliary decompression via the right intrahepatic bile ducts (Figure 4A). Complete obstruction at the level of the proximal bile duct was seen. US showed a well defined hyperechogenic cystic lesion of 4.4 cm, centrally in the liver. A solid calicification of 1 cm was situated inside the lesion. CT showed a cystic lesion with irregularly thickened wall, in conjunction with dilatated intrahepatic bile ducts (Figure 4B). Preoperative differential diagnosis of hepatobiliary cystadenoma, cystadenocarcinoma or choledochal cyst was made. The patient underwent hilar resection combined with extended right hemihepatectomy and biliary reconstruction using a hepaticojejunostomy. The diagnosis of hepatobiliary cystadenocarcinoma was made microscopically. The lesion was completely resected with microscopically free margins. The patient was still alive 59 mo after operation. Follow-up imaging showed no signs of local or distant tumor recurrence.

DISCUSSION

HBC are rare cystic neoplasms that may occur in the liver or in the extrahepatic biliary system. It is estimated that these cystic liver neoplasms comprise 5% of all cystic liver lesions[1]. Only a minority of patients with HBC develop symptoms, the most commonly reported symptoms are right abdominal pain, abdominal swelling, anorexia or nausea. Rarely, patients present with colicky pain or jaundice caused by obstruction of the biliary system. The differential diagnosis of HBC without symptoms includes simple cysts, choledochal cysts, hepatobiliary cystadenocarcinoma, hydatid cysts, abscess or haematoma. Obstructive jaundice is usually a presenting symptom of choledocholithiasis or malignant cholangiocarcinoma.

In the past 14 years, 13 patients have been diagnosed with HBC or cystadenocarcinoma in our institution. Five of these patients (38.5%) presented with biliary obstruction, which is usually caused by external biliary compression or by internal obstruction due to a mass inside the bile duct. Mucus hypersecretion in case of HBC or cystadenocarcinoma communicating with the bile ducts may also give rise to obstruction symptoms[4]. Three patients in this series presented with intermittent obstruction jaundice and two patients had continuous jaundice. In contrast, continuous biliary obstruction usually is the presenting symptom of patients with hilar cholangiocarcinoma.

Typically elevated cholestatic parameters in the blood are secondary to obstruction or compression of the biliary system. Some authors have suggested that serum CA-19-9 levels can be used for diagnosis or as a parameter of tumor activity during follow-up after resection[5,6]. CA-19-9, a serum marker normally synthesized by normal pancreatic and biliary ductal epithelium, is elevated both in benign biliary lesions and in malignant pancreatic carcinomas[7]. These serum markers, however, may be elevated in the presence of cholestasis and are therefore less reliable in the diagnosis of bile duct lesions. On imaging studies, distinction between HBC and simple cysts can be made by the presence of septations and irregularly thickened cyst walls with or without calcifications[8]. The most accurate imaging methods for detecting cystadenomas are abdominal ultrasound and CT-scan[9].

ERCP is useful, apart from establishing the diagnosis, in stenting the bile duct and decompressing the biliary system as was done in our patients. It can also help to differentiate between extraductal and intraductal obstructions.

However, it still remains difficult to differentiate between cystadenoma and cystadenocarcinoma by imaging methods[10]. In patients with cystic lesion at the hepatic hilum, diagnosis of choledochal cyst should be considered as well[11,12]. Liver hydatid disease, caused by Echinococcus granulosis, is usually asymptomatic and should also be considered as rupture of a hydatid cyst into the biliary tract, a well know complication, which also may give rise to colicky pain, cholangitis and obstructive jaundice. In these patients, ERCP is useful in detecting communication between the cystic lesion and the biliary system[13].

The use of percutaneous biopsy for preoperative diagnosis has no additional value considering the fact that it rarely produces a definitive diagnosis. There is also the additional risk of peritoneal dissemination in case of malignancy. Histopathological examination is required for definitive diagnosis. Microscopically, the linings of HBC are composed of a biliary type, mucus-secreting cuboidal or columnar epithelium. The underlying stroma shows presence of ovarian stroma in 85%-90% of HBC[14]. Distinction with simple liver cysts is made among other features, on the basis of the presence of subepithelial ovarian stroma in HBC. Simple liver cysts are composed of an outer layer of fibrous tissue and an inner lining of single columnar or cuboidal epithelium.

In the past, different treatment strategies such as partial resection, percutaneous aspiration or application of sclerosant agents inside the lesions have been applied to HBC. Patients treated with these techniques have shown high recurrence rates when compared to those who have undergone radical partial liver resection[15]. Another reason for resection is the possibility of malignant degeneration, although the precise risk remains unknown. In our series, cystadenocarcinoma was only seen in 2 of the 13 patients (15.4%) with HBC, which is comparable with the incidence in literature ranging from 5% to 25%[3,16]. The mean age of patients with cystadenocarcinomas has been reported to be approximately 17 years which is older than that of those without malignant degeneration[14]. The risk of malignant degeneration is also supported by the presence of benign epithelium in the wall of most cystadenocarcinomas[1]. Therefore, the treatment of choice should be radical surgical resection. The 5-year survival rate for cystadenocarcinomas after surgical resection ranges from 25% to 100%[17,18]. We observed no recurrence of cystadenocarcinomas during a mean follow-up of 56 mo (range 19-103 mo) by abdominal ultrasound or CT.

In conclusion, hepatobiliary cystadenoma or cystadenocarcinoma should be considered in patients with obstructive jaundice in the presence of a cystic liver lesion. ERCP and cross-sectional imaging techniques have a great value for establishing the diagnosis.

REFERENCES

1 **Ishak KG**, Willis GW, Cummins SD, Bullock AA. Biliary cystadenoma and cystadenocarcinoma: report of 14 cases and review of the literature. *Cancer* 1977; **39**: 322-338
2 **Devaney K**, Goodman ZD, Ishak KG. Hepatobiliary cystadenoma and cystadenocarcinoma. A light microscopic and immunohistochemical study of 70 patients. *Am J Surg Pathol* 1994; **18**: 1078-1091
3 **den Hoed PT**, Lameris H, Klooswijk B, IJzermans JN. Biliary cystadenoma: an uncommon cause of cholestatic jaundice. *Eur J Surg Oncol* 1999; **25**: 335-336
4 **Wang YJ**, Lee SD, Lai KH, Wang SS, Lo KJ. Primary biliary cystic tumors of the liver. *Am J Gastroenterol* 1993; **88**: 599-603
5 **Thomas JA**, Scriven MW, Puntis MC, Jasani B, Williams GT. Elevated serum CA 19-9 levels in hepatobiliary cystadenoma with mesenchymal stroma. Two case reports with immunohistochemical confirmation. *Cancer* 1992; **70**: 1841-1846
6 **Caturelli E**, Bisceglia M, Villani MR, de Maio G, Siena DA. CA 19-9 production by a cystadenoma with mesenchymal stroma of the common hepatic duct: a case report. *Liver* 1998; **18**: 221-224
7 **Steinberg W**. The clinical utility of the CA 19-9 tumor-associated antigen. *Am J Gastroenterol* 1990; **85**: 350-355
8 **Federle MP**, Filly RA, Moss AA. Cystic hepatic neoplasms: complementary roles of CT and sonography. *AJR Am J Roentgenol* 1981; **136**: 345-348
9 **Palacios E**, Shannon M, Solomon C, Guzman M. Biliary cystadenoma: ultrasound, CT, and MRI. *Gastrointest Radiol* 1990; **15**: 313-316
10 **Hai S**, Hirohashi K, Uenishi T, Yamamoto T, Shuto T, Tanaka H, Kubo S, Tanaka S, Kinoshita H. Surgical management of cystic hepatic neoplasms. *J Gastroenterol* 2003; **38**: 759-764
11 **de Vries JS**, de Vries S, Aronson DC, Bosman DK, Rauws EA, Bosma A, Heij HA, Gouma DJ, van Gulik TM. Choledochal cysts: age of presentation, symptoms, and late complications related to Todani's classification. *J Pediatr Surg* 2002; **37**: 1568-1573
12 **Park JH**, Lee DH, Kim HJ, Ko YT, Lim JW, Yang MH. Unilocular extrahepatic biliary cystadenoma mimicking choledochal cyst: a case report. *Korean J Radiol* 2004; **5**: 287-290
13 **Atli M**, Kama NA, Yuksek YN, Doganay M, Gozalan U, Kologlu M, Daglar G. Intrabiliary rupture of a hepatic hydatid cyst: associated clinical factors and proper management. *Arch Surg* 2001; **136**: 1249-1255
14 **Wheeler DA**, Edmondson HA. Cystadenoma with mesenchymal stroma (CMS) in the liver and bile ducts. A clinicopathologic study of 17 cases, 4 with malignant change. *Cancer* 1985; **56**: 1434-1445
15 **Organ B**, Petrek J. Biliary cystadenoma. *South Med J* 1984; **77**: 262-265
16 **Thomas KT**, Welch D, Trueblood A, Sulur P, Wise P, Gorden DL, Chari RS, Wright JK Jr, Washington K, Pinson CW. Effective treatment of biliary cystadenoma. *Ann Surg* 2005; **241**: 769-773; discussion 773-775
17 **Kubota E**, Katsumi K, Iida M, Kishimoto A, Ban Y, Nakata K, Takahashi N, Kobayashi K, Andoh K, Takamatsu S, Joh T. Biliary cystadenocarcinoma followed up as benign cystadenoma for 10 years. *J Gastroenterol* 2003; **38**: 278-282
18 **Lewis WD**, Jenkins RL, Rossi RL, Munson L, ReMine SG, Cady B, Braasch JW, McDermott WV. Surgical treatment of biliary cystadenoma. A report of 15 cases. *Arch Surg* 1988; **123**: 563-568

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CASE REPORT

Benign multicystic peritoneal mesothelioma: A case report and review of the literature

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Abstract

Benign multicystic peritoneal mesothelioma (BMPM) is a rare tumor that occurs mainly in women in their reproductive age. The pathogenesis of BMPM is unclear and a controversy regarding its neoplastic and reactive nature exists.

The biological behavior of BMPM is characterized by its slowly progressive process and high rate of recurrence after surgical resection. In addition this lesion does not present a strong tendency to transform into malignancy. Today approximately 130 cases have been reported.

We here report a 62-year-old woman who had diffuse abdominal pain, nausea and vomiting. Physical examination revealed a painful mass in her upper abdomen. She reported a mild dehydration, but the vital signs were normal. Peristaltic rushes, gurgles and high-pitched tinkles were audible. Upright plain abdominal film revealed small bowel loops with air-fluid levels. She was diagnosed having an incarcerated incisional hernia that resulted in intestinal obstruction. The patient underwent surgery during which a cystic mass of the right ovary measuring 6 cm x 5 cm x 4 cm, four small cysts of the small bowel (1 cm in diameter) and a cyst at the retroperitoneum measuring 11 cm x 10 cm x 3 cm were found. Complete resection of the lesion was performed. The patient had an uneventful recovery and had no recurrence two years after surgery.

Key words: Acute abdomen; Ovary; Peritoneum; Benign multicystic mesothelioma; Adenomatoid tumor

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INTRODUCTION

Benign multicystic peritoneal mesothelioma (BMPM), also known as multilocular peritoneal inclusion cysts, is an uncommon lesion arising from the peritoneal mesothelium that covers the serous cavity. This lesion occurs most frequently in women during their reproductive years[1-3] and is associated with a history of previous abdominal surgery[4], endometriosis[5,6] or pelvic inflammatory disease[4]. However, there are reports concerning men[7-9] or children[10-11], as well as rare extra-abdominal cases[12-14]. To date, approximately 130 cases have been reported[1,6,8,15-18]. While the origin of the disease is known, the pathogenesis and pathological differential diagnosis remain unclear and controversial. We here report a case of BMPM admitted to our department with a review of the literature.

CASE REPORT

A 62-year-old woman was referred to the Surgical Outpatient Department due to diffuse abdominal pain, nausea and vomiting for 24 h. On physical examination a painful mass in the upper part of a median subumbilical incision was palpated. She reported a history of hysterectomy five years earlier. A mild dehydration was noted, but the vital signs were normal. Peristaltic rushes, gurgles and high-pitched tinkles were audible. An upright plain abdominal film revealed small bowel loops with air-fluid levels. She was diagnosed having an incarcerated incisional hernia that resulted in intestinal obstruction. The patient underwent an emergency surgery during which a cystic mass of the right ovary measuring 6 cm × 5 cm × 4 cm, four small cysts of the small bowel (1 cm in diameter) and a cyst at the retroperitoneum measuring 11 cm × 10 cm × 3 cm were revealed. Complete resection of the lesion was performed. The pathology report was benign multicystic mesothelioma of the ovary, bowel and peritoneum (Figure 1). The patient had an uneventful recovery and was closely followed-up by US and CT. She remained free of symptoms and had no recurrence two years after surgery.

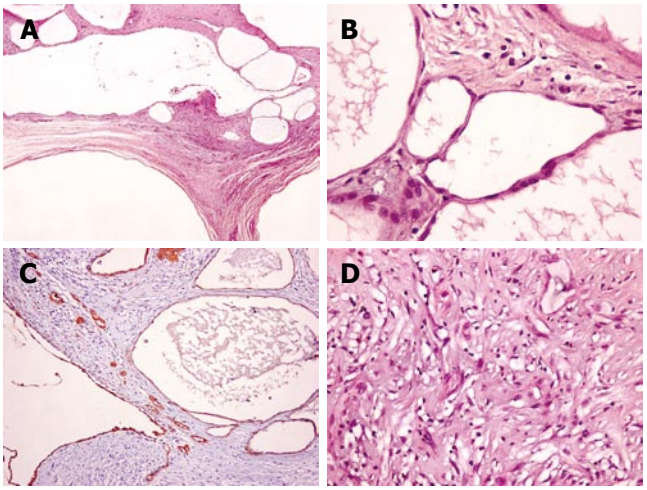

Figure 1 Histological and immunohistochemical findings in benign multicystic mesothelioma (**A**-**C**) and in ovary adenomatoid tumor (**D**) within collagenous stroma (**A**: HE x 20; **B**: HE x 400; **C**: AE1/AE3 x 100; **D**: HE x 400).

DISCUSSION

BMPM was first described in 1979 by Menemeyer and Smith[19]. Since then approximately 130 cases have been reported[20] and the information regarding BMPM is derived from a small number of patients from one institution or from isolated case reports. There are larger series reported from pathologist consultation files with patients from different institutions with incomplete clinical information and lack of long-time follow-up data[1,17]. Therefore, this disease is classified as an exceedingly rare medical entity, which challenges its origin, pathogenesis, diagnosis and therapy.

Origin

BMPM, a localized tumor arising from the epithelial and mesenchymal elements of the mesothelial cells, does not metastasize. It has a strong predilection for the surface of the pelvic viscera. When the tumor is found in the peritoneal cavity, lesions are found intimately attached to serosal surfaces of the intestine and omentum or in the retroperitoneal space, spleen and liver[21].

Pathogenesis

The pathogenesis of BMPM is a controversial entity. Some authors believe that the lesion is neoplastic, while others favor a reactive process[1,15-17,22]. The close relationship with inflammation, a history of prior surgery, endometriosis or uterine leiomyoma suggests that BMPM is probably a peculiar peritoneal reaction to chronic irritation stimuli, with mesothelial cell entrapment, reactive proliferation and cystic formation. Microscopic examination of the lesion reveals an inflammatory component in many cases. The close association of BMPM with familial Mediterranean fever characterized by periodic fever and peritonitis reinforces this assumption[23].

Other authors have proposed a neoplastic origin based on a slow but progressive growth of the untreated lesions, a marked tendency to recur after surgical resection, a low incidence of previous abdominal infection and a high disease-related mortality[1,15]. Malignant transformation of BMPM, an unusual occurrence, indicating a neoplastic nature underscoring the necessity of long-term follow-up has also been reported[20].

BMPM is rarely associated with adenomatoid tumor, another benign mesothelial lesion with its neoplastic and hyperplastic pathogenesis still argued[24]. Tumors with mixed histological features of multicystic mesothelioma and adenomatoid tumor have also been reported[24,25]. These facts indicate that a histogenetic relationship has led some authors to suggest that BMPM represents possibly a borderline lesion between an adenomatoid tumor and a malignant mesothelioma.

Pathological differential diagnosis

Pathological differential diagnosis includes a number of benign and malignant lesions that present as cystic or multicystic abdominal masses. Benign lesions include cystic lymphangioma (cystic hygroma)[8,15], cystic forms of endosalpingiosis[26,27], endometriosis[28], mullerian cysts involving the retroperitoneum[29], cystic adenomatoid tumors[30] and cystic mesonephric duct remnants[1]. Malignant lesions include malignant mesothelioma[17] and serous tumors involving the peritoneum[17]. Among the benign lesions, the most important differential diagnosis is BMPM from cystic lymphangioma and adenomatoid tumor. Cystic lymphangioma is restricted to the mesentery, omentum, mesocolon and retroperitoneum but rarely reported in the ovary. On gross examination the cystic component is often chylous and microscopic examination reveals bounds of smooth muscle and aggregates of lymphoid tissue. The cystic spaces are lined by a single layer of flattened endothelial cells which are immunoreactive to vascular markers (CD31, CD34, factor VIII, and VEGFR3). Cystic adenomatoid tumors are easily confused with BMPM on macroscopic and histological examination. However, the cystic component is usually accompanied with a recognizable solid component. Occasionally short papillae lined by mesothelial cells are seen. There are cases of tumors with mixed features of both adenomatoid tumor and BMPM[24,25], indicating that the two lesions are probably pathogenetically related. Cystic forms of endosalpingiosis differ from BMPM by the presence of a tubal type epithelium that may include peg cells, ciliated cells and/or secretory type cells. Blunt papilae and psammoma bodies may also present. The so-called "florid cystic endosalpingiosis" with multicystic involvement of either uterine or extrauterine sites appears to represent the extreme examples of this process[26,27]. Endometroid cysts typically containing dark chocolate-brown materials are composed histologically of endometrial stroma lined by endometrial-type epithelium. Commonly there is evidence of old or recent hemorrhage. Mullerian cyst is another benign condition that may be confused with BMPM. Nevertheless, as its name implies, it is composed of mullerian-type serous or mucinous epithelium containing smooth muscle fibers in the wall. The malignant conditions that mimic BMPM can be easily differentiated on the basis of malignant features including

cellular atypia, increased mitotic count, abnormal mitoses and destructive infiltration of the underlying stroma.

Diagnosis

In most cases, BMPM remains silent in abdominal cavity and may invade several underlying organs, and more rarely the retroperitoneum[31]. Sometimes BMPM is manifested as acute abdomen. In the case described here the first sign of the lesion was revealed only during surgery, which was performed on an emergency basis. In fact, most patients are diagnosed incidentally during examination or laparotomies for other reasons. The available modern imaging techniques, such as ultrasonography, computerized tomography and magnetic resonance imaging, can demonstrate the lesion. However a differential diagnosis is difficult to be made from other cystic neoplastic or inflammatory lesions arising from these anatomical areas[32-34]. From a clinical point of view, the differential diagnosis of BMPM from cystic tumors of ovaries is most important, since BMPM may be treated by local excision with preservation of the ovaries. Preoperative fine-needle aspiration biopsy of cystic lesions may help the differential diagnosis[35-37]. The diagnosis can be confirmed by electron microscopy and immunohistochemistry[38]. Benign cystic neoplasm with which BMPM is most likely confused is cystic lymphangioma, which occurs more commonly in males and may develop in children as well[17], but rarely recurs[8].

Therapy

Surgery is the only effective treatment for BMPM. Complete removal of the cystic lesion if possible, remains the mainstay of treatment and the only hope to avoid local recurrence. Aggressive surgical approaches including cytoreductive surgery with peritonectomy are recommended[39]. However, some investigators who support more conservative methods particularly claim that treatment options and timing of surgery should be made individually based on the benign morphologic pattern of the lesion[40]. The laparoscopic approach has also been attempted[41,42]. Recurrences occur more frequently in women and can be shown by CT guided cystic aspiration[43], and treated by hormonal therapy with anti-oestrogens[44] and gonadotrophin-releasing analogues[45], hyperthermic intraperitoneal chemotherapy[46,47], and sclerotherapy with tetracycline[48]. In any case the degree of success following these procedures varies. Adjuvant chemotherapy and radiotherapy are not indicated because these tumors have a prevailing benign character.

In conclusion, BMPM is a rare benign cystic tumor. Although its recurrence is high after surgical resection, it does not present a tendency to transform into malignancy. Two cases of malignant transformation[20,49] out of the approximately reported 130 cases cannot establish the incidence of this change, even if its neoplastic nature is supported. Finally, a prolonged systematic follow-up of these patients, perhaps for life, is required since the lesion usually reappears and further resection or other therapy may be indicated. Moreover, this strategy may lead the investigators to have a definite aspect concerning the biological behavior of the disease.

REFERENCES

1 **Weiss SW**, Tavassoli FA. Multicystic mesothelioma. An analysis of pathologic findings and biologic behavior in 37 cases. *Am J Surg Pathol* 1988; **12**: 737-746
2 **O'Neil JD**, Ros PR, Storm BL, Buck JL, Wilkinson EJ. Cystic mesothelioma of the peritoneum. *Radiology* 1989; **170**: 333-337
3 **Szöllósi A**, Ferenc C, Pintér T, Erényi A, Nagy A. [Benign cystic mesothelioma, a rare tumor of the peritoneum]. *Magy Seb* 2005; **58**: 35-37
4 **Tangjitgamol S**, Erlichman J, Northrup H, Malpica A, Wang X, Lee E, Kavanagh JJ. Benign multicystic peritoneal mesothelioma: cases reports in the family with diverticulosis and literature review. *Int J Gynecol Cancer* 2005; **15**: 1101-1107
5 **Groisman GM**, Kerner H. Multicystic mesothelioma with endometriosis. *Acta Obstet Gynecol Scand* 1992; **71**: 642-644
6 **Urbańczyk K**, Skotniczny K, Kuciński J, Friediger J. Mesothelial inclusion cysts (so-called benign cystic mesothelioma)--a clinicopathological analysis of six cases. *Pol J Pathol* 2005; **56**: 81-87
7 **Moore JH Jr**, Crum CP, Chandler JG, Feldman PS. Benign cystic mesothelioma. *Cancer* 1980; **45**: 2395-2399
8 **Carpenter HA**, Lancaster JR, Lee RA. Multilocular cysts of the peritoneum. *Mayo Clin Proc* 1982; **57**: 634-638
9 **Sienkowski IK**, Russell AJ, Dilly SA, Djazaeri B. Peritoneal cystic mesothelioma: an electron microscopic and immunohistochemical study of two male patients. *J Clin Pathol* 1986; **39**: 440-445
10 **Hanukoglu A**, Gewurtz G, Zaidel L, Krispin M, Fried D. Benign cystic mesothelioma of the peritoneum: the occurrence of an adult entity in a child. *Med Pediatr Oncol* 1992; **20**: 169-171
11 **McCullagh M**, Keen C, Dykes E. Cystic mesothelioma of the peritoneum: a rare cause of 'ascites' in children. *J Pediatr Surg* 1994; **29**: 1205-1207
12 **Ball NJ**, Urbanski SJ, Green FH, Kieser T. Pleural multicystic mesothelial proliferation. The so-called multicystic mesothelioma. *Am J Surg Pathol* 1990; **14**: 375-378
13 **Tobioka H**, Manabe K, Matsuoka S, Sano F, Mori M. Multicystic mesothelioma of the spermatic cord. *Histopathology* 1995; **27**: 479-481
14 **Lane TM**, Wilde M, Schofield J, Trotter GA. Benign cystic mesothelioma of the tunica vaginalis. *BJU Int* 1999; **84**: 533-534
15 **Katsube Y**, Mukai K, Silverberg SG. Cystic mesothelioma of the peritoneum: a report of five cases and review of the literature. *Cancer* 1982; **50**: 1615-1622
16 **McFadden DE**, Clement PB. Peritoneal inclusion cysts with mural mesothelial proliferation. A clinicopathological analysis of six cases. *Am J Surg Pathol* 1986; **10**: 844-854
17 **Ross MJ**, Welch WR, Scully RE. Multilocular peritoneal inclusion cysts (so-called cystic mesotheliomas). *Cancer* 1989; **64**: 1336-1346
18 **Sawh RN**, Malpica A, Deavers MT, Liu J, Silva EG. Benign cystic mesothelioma of the peritoneum: a clinicopathologic study of 17 cases and immunohistochemical analysis of estrogen and progesterone receptor status. *Hum Pathol* 2003; **34**: 369-374
19 **Mennemeyer R**, Smith M. Multicystic, peritoneal mesothelioma: a report with electron microscopy of a case mimicking intra-abdominal cystic hygroma (lymphangioma). *Cancer* 1979; **44**: 692-698
20 **González-Moreno S**, Yan H, Alcorn KW, Sugarbaker PH. Malignant transformation of "benign" cystic mesothelioma of the peritoneum. *J Surg Oncol* 2002; **79**: 243-251
21 **Flemming P**, Becker T, Klempnauer J, Högemann D, Kreft A, Kreipe HH. Benign cystic mesothelioma of the liver. *Am J Surg Pathol* 2002; **26**: 1523-1527
22 **Scucchi L**, Mingazzini P, Di Stefano D, Falchi M, Camilli A, Vecchione A. Two cases of "multicystic peritoneal mesothelioma": description and critical review of the literature. *Anticancer Res* 1994; **14**: 715-720
23 **Curgunlu A**, Karter Y, Tüfekci IB, Tunckale A, Karahasanoglu T. Benign cystic mesothelioma: a rare cause of ascites in a case with familial Mediterranean fever. *Clin Exp Rheumatol* 2003; **21**: S41-S43

24 **Chan JK**, Fong MH. Composite multicystic mesothelioma and adenomatoid tumour of the uterus: different morphological manifestations of the same process? *Histopathology* 1996; **29**: 375-377

25 **Zámecník M**, Gomolcák P. Composite multicystic mesothelioma and adenomatoid tumor of the ovary: additional observation suggesting common histogenesis of both lesions. *Cesk Patol* 2000; **36**: 160-162

26 **Clement PB**, Young RH. Florid cystic endosalpingiosis with tumor-like manifestations: a report of four cases including the first reported cases of transmural endosalpingiosis of the uterus. *Am J Surg Pathol* 1999; **23**: 166-175

27 **Heatley MK**, Russell P. Florid cystic endosalpingiosis of the uterus. *J Clin Pathol* 2001; **54**: 399-400

28 **Bell DA**, Scully RE. Benign and borderline serous lesions of the peritoneum in women. *Pathol Annu* 1989; **24** Pt 2: 1-21

29 **de Peralta MN**, Delahoussaye PM, Tornos CS, Silva EG. Benign retroperitoneal cysts of müllerian type: a clinicopathologic study of three cases and review of the literature. *Int J Gynecol Pathol* 1994; **13**: 273-278

30 **Bisset DL**, Morris JA, Fox H. Giant cystic adenomatoid tumour (mesothelioma) of the uterus. *Histopathology* 1988; **12**: 555-558

31 **Villaschi S**, Autelitano F, Santeusanio G, Balistreri P. Cystic mesothelioma of the peritoneum. A report of three cases. *Am J Clin Pathol* 1990; **94**: 758-761

32 **Romero JA**, Kim EE, Kudelka AP, Edwards CL, Kavanagh JJ. MRI of recurrent cystic mesothelioma: differential diagnosis of cystic pelvic masses. *Gynecol Oncol* 1994; **54**: 377-380

33 **Ozgen A**, Akata D, Akhan O, Tez M, Gedikoglu G, Ozmen MN. Giant benign cystic peritoneal mesothelioma: US, CT, and MRI findings. *Abdom Imaging* 1998; **23**: 502-504

34 **Abdullahi H**, Fawzi H. Gynaecological presentation of benign multicystic mesothelioma. *J Obstet Gynaecol* 2003; **23**: 576

35 **Tao LC**. Aspiration biopsy cytology of mesothelioma. *Diagn Cytopathol* 1989; **5**: 14-21

36 **Bhandarkar DS**, Smith VJ, Evans DA, Taylor TV. Benign cystic peritoneal mesothelioma. *J Clin Pathol* 1993; **46**: 867-868

37 **van Ruth S**, Bronkhorst MW, van Coevorden F, Zoetmulder FA. Peritoneal benign cystic mesothelioma: a case report and review of the literature. *Eur J Surg Oncol* 2002; **28**: 192-195

38 **Petrou G**, Macindoe R, Deane S. Benign cystic mesothelioma in a 60-year-old woman after cholecystectomy. *ANZ J Surg* 2001; **71**: 615-618

39 **Sethna K**, Mohamed F, Marchettini P, Elias D, Sugarbaker PH. Peritoneal cystic mesothelioma: a case series. *Tumori* 2003; **89**: 31-35

40 **Meister** T, Birkfellner T, Poremba C, Becker JC, Menzel J, Domschke W, Lerch MM. Papillary mesothelioma of the peritoneum in the absence of asbestos exposure. *Z Gastroenterol* 2003; **41**: 329-332

41 **Ricci F**, Borzellino G, Ghimenton C, Cordiano C. Benign cystic mesothelioma in a male patient: surgical treatment by the laparoscopic route. *Surg Laparosc Endosc* 1995; **5**: 157-160

42 **Vara-Thorbeck C**, Toscano-Mendez R. Peritoneal cystic mesothelioma. *Surg Endosc* 2002; **16**: 220

43 **Inman DS**, Lambert AW, Wilkins DC. Multicystic peritoneal inclusion cysts: the use of CT guided drainage for symptom control. *Ann R Coll Surg Engl* 2000; **82**: 196-197

44 **Letterie GS**, Yon JL. The antiestrogen tamoxifen in the treatment of recurrent benign cystic mesothelioma. *Gynecol Oncol* 1998; **70**: 131-133

45 **Letterie GS**, Yon JL. Use of a long-acting GnRH agonist for benign cystic mesothelioma. *Obstet Gynecol* 1995; **85**: 901-903

46 **Ma GY**, Bartlett DL, Reed E, Figg WD, Lush RM, Lee KB, Libutti SK, Alexander HR. Continuous hyperthermic peritoneal perfusion with cisplatin for the treatment of peritoneal mesothelioma. *Cancer J Sci Am* 1997; **3**: 174-179

47 **Park BJ**, Alexander HR, Libutti SK, Wu P, Royalty D, Kranda KC, Bartlett DL. Treatment of primary peritoneal mesothelioma by continuous hyperthermic peritoneal perfusion (CHPP). *Ann Surg Oncol* 1999; **6**: 582-590

48 **Benson RC Jr**, Williams TH. Peritoneal cystic mesothelioma: successful treatment of a difficult disease. *J Urol* 1990; **143**: 347-348

49 **Hejmadi R**, Ganesan R, Kamal NG. Malignant transformation of a well-differentiated peritoneal papillary mesothelioma. *Acta Cytol* 2003; **47**: 517-518

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Activation of c-Yes in hepatocellular carcinoma: A preliminary study

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TO THE EDITOR

Hepatocellular carcinoma (HCC) is thought to develop through a multistep process[1]. A long history of viral hepatitis or prolonged exposure to environmental toxins predisposes liver cells to mutations of the genes critical in the control of hepatocyte growth. In fact, both activation of cellular oncogenes and inactivation of tumor-suppressor genes are involved in the development of HCC. Activation of oncogenes by hepatitis virus integration has been shown in the woodchuck animal model[2], although the significance of this finding in human hepatocarcinogenesis is still under investigation. Tyrosine kinases, though a minor class of cellular protein, represent a major class of oncogenes. These tyrosine kinases are classified into two major groups[3,4]. The first is receptor-type protein tyrosine kinases, the second is non-receptor type tyrosine kinases. The main representatives of the latter group are non-receptor-linked and membrane-associated Src family tyrosine kinases. At least nine Src-related tyrosine kinases have been identified thus far, including c-Yes, c-Src, c-Lck, c-Fyn, c-Hck, c-Lyn, c-Blk, c-Fgr and c-Yrk protooncogene products. The cellular oncogene c-Yes, a member of the Src family, encodes a 62-kilodalton, cytoplasmic and membrane-associated protein-tyrosine kinase[5]. c-Yes expression and its kinase activity have been shown to be increased in colorectal cancer[6,7], melanoma[8] and metastatic liver cancer[9]. However, the activation of c-Yes in human HCC has not yet been investigated at all. In the present study, we determined the activity of c-Yes both in the normal liver (NL) tissues and in chronic hepatitis (CH), tumorous (T) and adjacent nontumorous (N) cirrhotic liver tissues. This is the first report on the activity of c-Yes in various liver diseases including HCC.

Tissue samples, including the tumorous and surrounding nontumorous cirrhotic tissues, were obtained during surgery from 9 patients with HCC (7 males and 2 females; mean age, 68.2 ± 4.4 years; range, 59-73 years). All the patients were positive for hepatitis C virus (HCV), as determined by the reverse-transcriptase polymerase chain reaction method (Amplicor, Roche Diagnostics Ltd). The number of patients with well-, moderately- and poorly-differentiated HCC was 2, 5 and 2, respectively. The fibrosis stage of the surrounding liver tissues was assessed as cirrhosis in all the HCC cases. Liver tissue samples from three patients with HCV-induced CH were obtained by liver biopsy. During surgery, two normal liver tissue samples were also obtained from patients with liver metastases of colon cancer. The serum samples of these patients with NL were negative for HCV and hepatitis B virus (HBV).

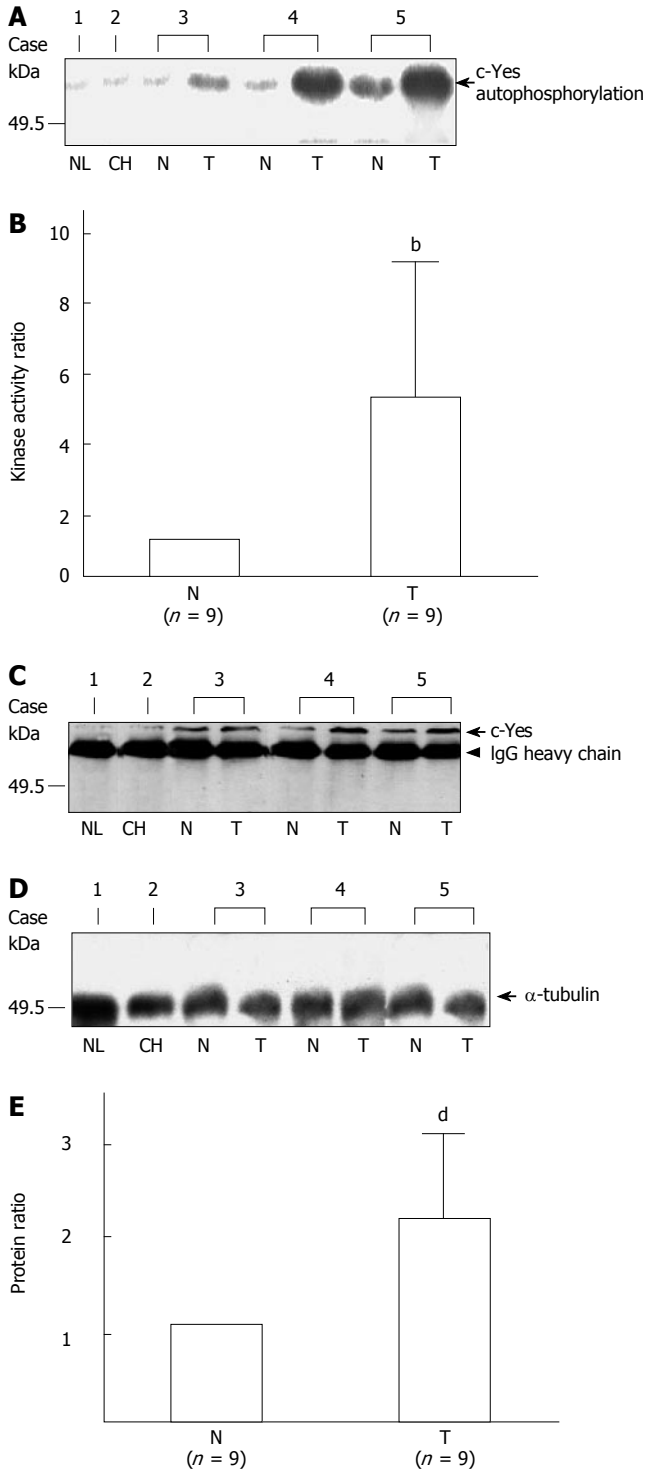

Figure 1 Activities and levels of c-Yes kinase in different liver tissues. **A**: Activity of c-Yes in normal liver (NL), chronic hepatitis (CH), nontumorous cirrhotic (N) and tumorous (T) tissues. The arrow indicates the band corresponding to c-Yes autophosphorylation; **B**: Relative levels of total c-Yes activity (mean ± SE, Student's *t*-test, $^{b}P < 0.001$); **C**: Levels of c-Yes in the NL, CH, N, and T portions of HCC. The arrow and arrowhead indicate the bands corresponding to c-Yes protein and the heavy-chain of the c-Yes antibody, respectively; **D**: Level of α-tubulin in lysate containing 100 μg of cellular protein used in the immunoprecipitation (arrow); **E**: Relative levels of c-Yes protein in T and N tissues (mean ± SE, Student's *t*-test, $^{d}P < 0.01$).

To determine the protein kinase activity of c-Yes in the NL, CH, N and T portions of HCC, we prepared lysates containing 100 μg of cellular protein of tissue samples, precipitated the protein with a monoclonal antibody specific for c-Yes [MAb (3H9), Wako Pure Chemical Co, Tokyo], and measured the autophosphorylation of c-Yes using an *in vitro* protein kinase assay described previously[6,8]. Activities of c-Yes in the representative results of NL (patient 1), CH (patient 2), N and T tissues (patients 3-5), respectively, are shown in Figure 1A. When the kinase level of N tissues was used as a reference level ($n = 1$), c-Yes activity ratio in the T tissues ($n = 9$) was 5.0 ± 3.7 times higher than that in the N tissues, as measured by autophosphorylation (Student's *t*-test, $P < 0.001$) (Figure 1B). Patients 3-5 were classified as well–differentiated, moderately-differentiated and poorly-differentiated HCC, respectively. In addition, the c-Yes activity in all NL and CH tissues used in this study was very low as measured by autophosphorylation of c-Yes. Thus, c-Yes activity was notably high in human liver tissues with malignancy. In addition, our results showed that although the kinase activity of c-Yes was very low in NL and CH, it was already activated in liver cirrhosis (nontumorous cirrhotic tissues). Because the kinase activity of c-Yes was already activated at the preneoplastic stage (cirrhosis), *i.e.*, before the development of HCC, these data suggest that HCV-induced liver cirrhosis is a precancerous condition.

Protein levels of c-Yes were determined by Western blot analysis. Immunoprecipitates used for the protein kinase assays were also applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose membranes. As shown in Figure 1C, two proteins were detected in each lane. The upper protein was c-Yes, and the lower one was the heavy chain of the c-Yes monoclonal antibody. Although the level of c-Yes was very low in NL and CH, it was elevated in N and T tissues of HCC (Figure 1C). As an internal control, the amount of α-tubulin in lysate containing 100μg of cellular protein used in the immunoprecipitation was almost the same in each lane (Figure 1D). When the protein level of N tissues was used as a reference level ($n = 1$), the level of c-Yes protein in T tissues ($n = 9$) was 2.1 ± 0.84 times higher than that in the surrounding N tissues from the same patients (Student's *t*-test, $^{d}P < 0.01$) (Figure 1E). The ratio (T tissue *vs* surrounding N tissue) of the protein level of c-Yes was smaller than that of the kinase activity. These data suggest that the high c-Yes kinase activity in the HCC is probably caused not only by an increase of c-Yes protein but also by an increase of the enzyme activity.

In conclusion, activation of the protooncogene product c-Yes may play a significant role in the malignant transformation of hepatocytes. The suppression of c-Yes kinase activity may offer a novel strategy for overcoming the development and invasion of HCC. Further studies are necessary to investigate such processes.

REFERENCES

1 **Sugimura T**. Multistep carcinogenesis: a 1992 perspective. *Science* 1992; **258**: 603-607
2 **Fourel G**, Trepo C, Bougueleret L, Henglein B, Ponzetto A, Tiollais P, Buendia MA. Frequent activation of N-myc genes by hepadnavirus insertion in woodchuck liver tumours. *Nature* 1990; **347**: 294-298
3 **Neet K**, Hunter T. Vertebrate non-receptor protein-tyrosine kinase families. *Genes Cells* 1996; **1**: 147-169

4 **Hunter T**. The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. *Philos Trans R Soc Lond B Biol Sci* 1998; **353**: 583-605
5 **Bolen JB**. Nonreceptor tyrosine protein kinases. *Oncogene* 1993; **8**: 2025-2031
6 **Park J**, Meisler AI, Cartwright CA. c-Yes tyrosine kinase activity in human colon carcinoma. *Oncogene* 1993; **8**: 2627-2635
7 **Hirsch CL**, Smith-Windsor EL, Bonham K. Src family kinase members have a common response to histone deacetylase inhibitors in human colon cancer cells. *Int J Cancer* 2006; **118**: 547-554
8 **Loganzo F Jr**, Dosik JS, Zhao Y, Vidal MJ, Nanus DM, Sudol M, Albino AP. Elevated expression of protein tyrosine kinase c-Yes, but not c-Src, in human malignant melanoma. *Oncogene* 1993; **8**: 2637-2644
9 **Han NM**, Curley SA, Gallick GE. Differential activation of pp60(c-src) and pp62(c-yes) in human colorectal carcinoma liver metastases. *Clin Cancer Res* 1996; **2**: 1397-1404

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LETTERS TO THE EDITOR

Unilateral leg edema in a cirrhotic patient with tense ascites

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TO THE EDITOR

A 61 year old man with cirrhosis and hepatocellular carcinoma developed on the background of chronic hepatitis B was admitted because of acute and gradually intensified right thigh pain and swelling, which, within a few hours, was expanded to his right foot. The patient, due to ascites refractory to diuretics, was almost weekly subjected to large volume paracentesis of ascitic fluid for the last six months; meanwhile he has developed a left inguinal, a right femoral and an umbilical hernia. It should be noted that 48 h before admission, the patient was referred to the emergency room because of dyspnoea and 5 L of ascitic fluid were removed.

Physical examination was notable of tense ascites and his right lower extremity was profoundly more edematous (thigh, calf and foot) compared to his left leg (Figure 1), without signs of inflammation. Inguinal lymph nodes were not enlarged, Homan's sign was absent, and peripheral arteries were palpable. There was also a left inguinal hernia and an umbilical hernia containing fluid in the sac, while there was no edema in the scrotum.

The patient was initially subjected to duplex ultrasonography of his right leg, which turned negative for deep venous thrombosis. Subsequently, a CT scan of the right thigh and abdomen was performed, revealing an enormous fluid collection within the peritoneal cavity (Figure 2A) and a notable fluid collection within the lateral aspect of the right thigh (Figure 2C). The fluid in the right thigh exhibited approximately the same density with ascitic fluid (Figure 2A and C). No masses were detected in the pelvis.

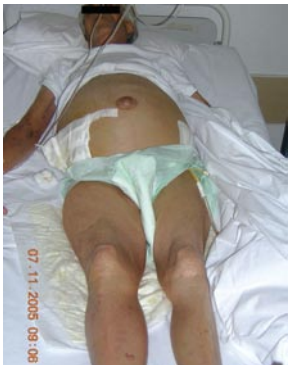

Figure 1 Significant edema of the right leg without signs of inflammation in our cirrhotic patient with tense ascites.

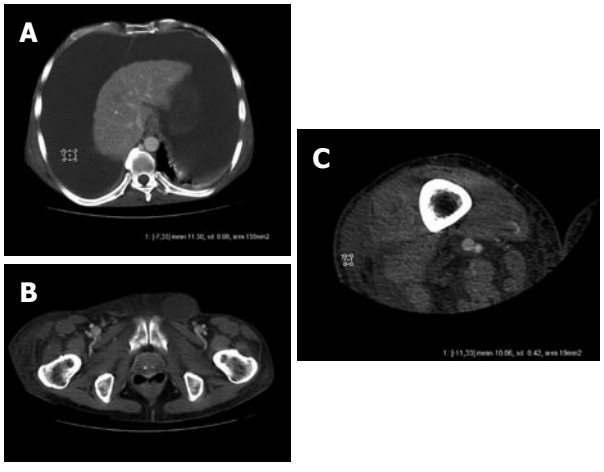

Figure 2 **A**: Enormous fluid collection within the peritoneal cavity. The fluid density was measured to be 11.5 Hounsfield Units; **B**: No masses were detected in the pelvis, whereas there was fluid collection into the left inguinal canal and no evidence of fluid collection into the right inguinal canal; **C**: Fluid collection within the lateral aspect of the right thigh with a density of 10.06 Hounsfield Units.

In addition, fluid was also seen in the left inguinal canal, but not in the scrotum, while no hernia was seen in the right femoral ring (Figure 2B). What could be the etiology

of the patient's right lower extremity edema?

According to the diagnostic evaluation performed, the possibility of deep venous thrombosis of the right leg was practically excluded based on negative duplex, increased international normalized ratio (INR) (2.2) and low platelet count (70 000/mm^3) that our patient exhibited in the text of his cirrhosis. Also any mechanical reasons that could induce unilateral venous or lymphatic stasis were ruled out. However, according to the CT scan findings, it seemed that the patient's lower extremity edema was caused by accumulation of ascitic fluid, which somehow passed from the peritoneal cavity to the right thigh. Interestingly, the known right femoral hernia was detected neither by physical examination, nor by CT scan, tempting us to speculate that drainage of fluid contained within the femoral hernial sac to the thigh might have occurred.

Asking in more detail the patient's history we were informed that in his prior admission to the emergency room (48 h ago), he was subjected to right femoral vein puncture for blood sampling. A potential mechanism of ascitic fluid accumulation in the right thigh could be the injury of the right femoral hernial sac during femoral vein puncture. The femoral hernial sac and its content pass through the femoral ring and are generally localized between the external femoral vein and Gimbernat's lacunar ligament medially. These hernias, having penetrated Scarpa's triangle, through stretching of the cribrate lamina, are visible subcutaneously in the thigh. Reasonably, perforation of the femoral hernial sac would permit the drainage of ascitic fluid to the subcutaneous tissue of the right thigh, forced by the high intraabdominal pressure, whereas the scrotum would remain unaffected (unlike the case of an inguinal hernia perforation). The patient was subjected to repeated large volume paracentesis and along with ascites improvement his right leg edema was significantly decreased.

This case presents a rare complication of femoral vein puncture in a patient with tense ascites, which raised diagnostic dilemmas and led to expensive diagnostic evaluation. Clinicians handling cirrhotic patients should be aware of this potential complication and perform femoral vein or artery puncture with caution or ideally under scan control in case of coexistence of ascites and femoral or inguinal hernias.

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LETTERS TO THE EDITOR

Diagnostic dilemma between intestinal Behçet disease and inflammatory bowel disease with pyoderma gangrenosum

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TO THE EDITOR

I have read with great interest the very recent article titled "Intestinal Behçet's disease with pyoderma gangrenosum: A case report" of Nakamura T *et al* that was published in your journal. The authors stated that they presented a very rare case of intestinal Behçet's disease with pyoderma gangrenosum in a 16-year old patient. However, I would like to make some important contributions and suggestions to the presented case and have a few questions to ask the authors.

First, the exact diagnosis of Behçet disease in a single case depends first on the recognition of a characteristic set of sufficient symptoms and/or signs to allow the physician to diagnose with various levels of certainty from "complete or definitive" Behçet disease to "suspected or possible" Behçet disease[1] based on the clinical sign constellation in Japanese Behçet Disease Research Committee Criteria, the preferred diagnostic criteria before 1989[2]. According to the criteria, such a "complete" or "definitive" diagnosis of Behçet disease needs either at least three major criteria or two major criteria (one of which is ocular disease) or two major criteria associated with 2 minor criteria. This means that the correct diagnosis of Behçet disease can be established only on the basis of aforementioned strict rules that need the complete fulfillment of the criteria with careful differential diagnosis from other etiologies. Therefore, the authors' statement that "she was diagnosed with intestinal Behçet' s disease by the presence of cutaneous pathergy together with two major criteria (oral and genital aphthoses) and one minor criterion (gastrointestinal manifestations) in 1984" cannot be accepted as the presented case does not fulfill the requirements of "Japanese Behçet Disease Research Committee Criteria", the preferred diagnostic criteria in that period, in which cutaneous pathergy is not included in the major or minor criteria. Although oral aphthous and genital ulcerations with a positive pathergy test of the presented case meet the diagnostic criteria of International Study Group[3], published in 1990, however, diagnosis and surgical intervention of the presented case with "incomplete diagnosis" were made in 1989. In other words, the case can be accepted as "suspected" Behçet disease according to the Japanese criteria, provided that other etiologies for these manifestations have been strictly ruled out including inflammatory bowel disease (IBD). Therefore, the authors should first clarify this diagnostic confusion.

Second, the presented case was stated to have colitis involving the entire colon demonstrated by colonoscopy. However, typical intestinal involvement in Behçet disease is characterized mainly by changes in the small intestine or ulcerative lesions at the terminal ileum or cecum, resulting in various digestive symptoms[4]. I think the differential diagnosis was not performed strictly enough in this single case as the aforementioned symptoms are seen in IBD, namely Crohn's disease and ulcerative colitis that need careful evaluation. Although the resected specimen demonstrated severe inflammation with neutrophil accumulation, this is a general finding in inflammatory diseases and intestinal Behçet disease is often indistinguishable from IBD by histological evaluation. Therefore, such a general finding cannot be used as the histological hallmark of intestinal Behçet disease as its diagnosis is based on the presence of deep colonic ulcerations frequently situated in healthy mucosa along with the presence of an adjacent non-specific inflammatory infiltrate affecting the entire colonic wall that is characterized by leukocytoclastic vasculitis and perivasculitis of the arteries and veins with signs of fibrinoid necrosis. Moreover, there is not any knowledge in the presented article about the presence or absence of granulomatous or non-granulomatous changes or caseation with or without confluent (diffuse) or skipping

(segmental) lesions (*i.e.*, mural thickening) that strongly need strict differential diagnosis of intestinal Behçet disease from IBD. Indeed, ulcerative colon, for instance, is restricted to the colon, and exhibits proximal extension over time with full thickness involvement in case of toxic megacolon[5]. Therefore, nothing in the article can exclude these questions in that single case that seems to need clarification.

Third, the authors stated that the patient received 20 mg oral prednisolone treatment for a month, though cutaneous and intestinal lesions of the girl were poorly controlled. However, to our knowledge, corticosteroids (CSs) alone are not used for the treatment[1] and management[6] of Behçet disease at least in the used dose, and no study has found that CSs alone are effective on any symptom or sign as well as on any etiological factors including cutaneous and intestinal lesions that are accused for the pathogenesis of Behçet disease[7-13]. Moreover, CSs are of little value for the maintenance of remission. Indeed, the results of Mat C *et al*[14] are important and demonstrate that CSs alone for about a six- month duration are not effective even on oromucocutaneous symptoms despite the well-known underlying vasculitic pathology of the condition. Because most of the treatments have been shown to work in Behçet disease or in IBD, the used dose of CSs seems not sufficient for such a severe intestinal involvement in the present case and therefore, the patient should have been treated first with a higher dose of CSs (1-2 mg/kg prednisolone per day) for a short time with some other immunomodulating/immunosuppressive agents or their combinations with cyclophosphamide, chlorambucil, colchicine, dapsone, cyclosporine and especially azathioprine that could be used in that period for the management and treatment of complete or incomplete Behçet disease patients with or without intestinal findings, to induce rapid and durable remission of intestinal attacks before the decision of an invasive surgery (total colectomy) is made[15-21]. In other words, whether the patient is supposed to suffer from Behçet disease or IBD, the indication for surgical treatment can be made upon failure of sufficient and appropriate medical treatment or based on the severe complications such as abscess, fistula formation and iatrogenic perforation. Although systemic CSs may be useful in the early stages of severe inflammatory attacks, they are of limited value for long-term management of serious involvements whereas sustained remission can be accomplished only by the immunomodulatory agents. For instance, azathioprine or chlorambucil associated with high dose CSs should have been tried initially for the girl to obtain possible remission with long-term steroid-sparing effects. Similarly, cyclophosphamide- and cyclosporine-CS combinations have also been used between 1971 and 1989 for various symptoms of Behçet disease, even in cases resistant to conventional therapy[22-28]. Furthermore, Sanderson[29] has evaluated chronic IBD in children including Crohn's disease, ulcerative colitis, indeterminate colitis and Behçet's colitis in the review in that period and stated that treatment with drugs (sulphasalazine, steroids, azathioprine) and elemental diet are helpful and concluded that the prognosis of chronic IBD in childhood is good. More importantly, sulphasalazine with or without cyclosporine and CSs has successfully been used for the treatment of both intestinal Behçet disease and IBD[30-32].

Fourth, the authors further stated that intractable ulceration of the left foot surprisingly disappeared postoperatively after total colectomy within two weeks with no relapse of pyoderma gangrenosum for 10 years afterwards, suggesting a close relationship between pyoderma gangrenosum and intestinal Behçet disease. I do not agree with the authors on this regard and I believe that if the patient had really had Behçet disease, pyoderma gangrenosum or any other kind of cutaneous manifestations might have developed in its due course as the disease is known to be most active during the second and third decades of life. Indeed, the authors further stated in their paper that pyoderma gangrenosum might respond to surgical resection of the associated diseases, such as ulcerative colitis and Crohn's disease (see discussion section, second paragraph), and then stated in the following sentences that pyoderma gangrenosum in the present case rapidly improved after total colectomy. I agree on this regard and fortunately, total colectomy results in cure of IBD with reasonable long-term benefit in many cases. Therefore, both the authors' statements in their paper and the literature again indicate that the girl had an IBD with or without Behçet disease.

Although aphthous punched-out ulcerations may be found in about one-tenth of Behçet disease patients, they occur most frequently in the terminal ileum and cecum[33] and this finding alone is still not sufficient for the diagnosis of Behçet disease as the case should strictly fulfill the whole systemic diagnostic criteria as stated above and aphthae in the colon may be seen not only in Behçet disease, but also, for instance, in Crohn's disease[34]. Moreover, a very recent study has demonstrated that none of the clinical parameters of a total of 162 consecutive adult patients with diagnosis of IBD consisting of Crohn's disease and ulcerative colitis fulfill the Behçet disease diagnostic criteria[35]. In other words, Behçet disease was diagnosed in none of the patients even though they had various symptoms such as intestinal involvement, pyoderma gangrenosum, articular disease, skin lesions, and oral ulcer with or without HLA-B51 positivity. Furthermore, such case reports with identical and perplexing clinical presentations as compared to those of Nakamura *et al*, are still present in the literature, describing association of both diseases[32,36,37].

In conclusion, although I agree that the gastrointestinal and systemic features of Behçet disease and IBD overlap to a considerable extent that need addressing both in terms of increasing our understanding of pathogenesis and improving therapy, they are generally viewed as two distinct diseases, and oral aphthous ulcerations, genital ulcerations, papulopustular lesions or pyoderma gangrenosum on the leg with or without pathergy test positivity are all encountered findings during the course of IBD. In addition, constellation of findings for the diagnosis of Behçet disease is applicable only in the absence of other clinical explanations such as IBD. Therefore, the absence of sufficient symptoms to diagnose this young girl as "complete" or "definitive" Behçet disease

along with the presence of active colitis and ulcerations involving the entire alimentary tract, mainly colon in that case, reaching up to the terminal ileum associated with a rapid disappearance of intractable foot ulceration after surgery with the lack of demonstrated thrombotic or leukocytoclastic vasculitis (typical hallmark of Behçet disease) on colonic histopathological examination in the presented case, strongly indicates a diagnosis of pyoderma gangrenosum associated with IBD characterized by Behçet disease-like clinical presentation[38-40]. Consequently, I suggest that the authors of the present case perform HLA analysis in their patient. If it reveals HLA-B51 positivity, then the very unusual diagnosis of classified or unclassified IBD associated with Behçet disease can be made in that case. If it reveals HLA-B27 positivity, then the patient can be classified in that case as HLAB-27 positive IBD. Even if the patient is still to be accepted as having intestinal Behçet disease, it seems that the rules for the treatment of such an involvement have not been followed before the surgery.

REFERENCES

1 **Evereklioglu C**. Current concepts in the etiology and treatment of Behçet disease. *Surv Ophthalmol* 2005; **50**: 297-350
2 Skin hypersensitivity to streptococcal antigens and the induction of systemic symptoms by the antigens in Behçet's disease--a multicenter study. The Behcet's Disease Research Committee of Japan. *J Rheumatol* 1989; **16**: 506-511
3 Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet* 1990; **335**: 1078-1080
4 **Marchetti F**, Trevisiol C, Ventura A. Intestinal involvement in children with Behçet's disease. *Lancet* 2002; **359**: 2115
5 **Geboes K**, Collins S. Structural abnormalities of the nervous system in Crohn's disease and ulcerative colitis. *Neurogastroenterol Motil* 1998; **10**: 189-202
6 **Evereklioglu C**. Managing the symptoms of Behçet's disease. *Expert Opin Pharmacother* 2004; **5**: 317-328
7 **Evereklioglu C**, Yurekli M, Er H, Ozbek E, Hazneci E, Cekmen M, Inaloz HS. Increased plasma adrenomedullin levels in patients with Behçet's disease. *Dermatology* 2000; **201**: 312-315
8 **Evereklioglu C**, Turkoz Y, Er H, Inaloz HS, Ozbek E, Cekmen M. Increased nitric oxide production in patients with Behçet's disease: is it a new activity marker? *J Am Acad Dermatol* 2002; **46**: 50-54
9 **Evereklioglu C**, Er H, Türköz Y, Cekmen M. Serum levels of TNF-alpha, sIL-2R, IL-6, and IL-8 are increased and associated with elevated lipid peroxidation in patients with Behçet's disease. *Mediators Inflamm* 2002; **11**: 87-93
10 **Evereklioglu C**, Inalöz HS, Kirtak N, Doganay S, Bülbül M, Ozerol E, Er H, Ozbek E. Serum leptin concentration is increased in patients with Behçet's syndrome and is correlated with disease activity. *Br J Dermatol* 2002; **147**: 331-336
11 **Evereklioglu C**, Ozbek E, Er H, Cekmen M, Yürekli M. Urinary adrenomedullin levels are increased and correlated with plasma concentrations in patients with Behçet's syndrome. *Int J Urol* 2002; **9**: 296-303
12 **Evereklioglu C**, Cekmen M, Ozkiriş A, Karabaş L, Caliş M. The pathophysiological significance of red blood cell nitric oxide concentrations in inflammatory Behçet's disease. *Mediators Inflamm* 2003; **12**: 255-256
13 **Evereklioglu C**, Ozbek E, Cekmen M, Mehmet N, Duygulu F, Ozkiris A, Calip M, Er H, Turkoz Y. Urinary nitric oxide levels are increased and correlated with plasma concentrations in patients with Behçet's disease: is it a new urinary activity marker? *Nephrology* (Carlton) 2003; **8**: 231-238
14 **Mat C**, Yurdakul S, Uysal S, Gogus F, Ozyazgan Y, Uysal O, Fresko I, Yazici H. A double-blind trial of depot corticosteroids in Behçet's syndrome. *Rheumatology* (Oxford) 2006; **45**: 348-352
15 **Témime P**, Bérard P, Privat Y, Oddoze L, Marchand JP. [Behcet syndrome treated with chlorambucil]. *Bull Soc Fr Dermatol Syphiligr* 1969; **76**: 433-434
16 **Nethercott J**, Lester RS. Azathioprine therapy in incomplete Behcet syndrome. *Arch Dermatol* 1974; **110**: 432-434
17 **Firat T**. Results of immunosuppressive treatment in Bechçet's disease: report of 55 cases. *Ann Ophthalmol* 1978; **10**: 1421-1423
18 **Kadoya M**, Okinami S. [Treatment of Behçet's disease with immunosuppressive agents and colchicine (author's transl)]. *Nihon Ganka Gakkai Zasshi* 1979; **83**: 200-206
19 **Psilas K**, Sideroglou P. [Treatment of the Adamantiades-Behçet syndrome using an immunosuppressive agent (azathioprine) and prednisolone]. *Bull Mem Soc Fr Ophtalmol* 1983; **95**: 417-422
20 **Abdalla MI**, el-D Bahoat N. Long-lasting remission of Behcet's disease after chlorambucil therapy. *Br J Ophthalmol* 1973; **57**: 706-711
21 **Plotkin GR**, Patel BR, Shah VN. Behçet's syndrome complicated by cutaneous leukocytoclastic vasculitis. Response to prednisone and chlorambucil. *Arch Intern Med* 1985; **145**: 1913-1915
22 **De Bast C**. [Behçet's syndrome: treatment with prednisolone-cyclophosphamide association]. *Arch Belg Dermatol Syphiligr* 1971; **27**: 299-304
23 **Oniki S**, Kurakazu K, Kawata K. [Treatment of Behçet's disease with cyclophosphamide]. *Nihon Ganka Gakkai Zasshi* 1973; **77**: 508-515
24 **Davatchi F**, Baygan F, Chams H, Chams C. Cyclophosphamide in the treatment of the ocular manifestations of Behçet's disease. *J Rheumatol* 1984; **11**: 404-405
25 **Nussenblatt RB**, Palestine AG. Cyclosporine: immunology, pharmacology and therapeutic uses. *Surv Ophthalmol* 1986; **31**: 159-169
26 **Le Thi Huong Du**, Vidailhet MJ, Wechsler B, Piette JC, Ziza JM, Chapelon C, Bletry O, Godeau P. [Value of the cyclophosphamide bolus in severe systemic diseases. Preliminary results]. *Presse Med* 1988; **17**: 524-527
27 **BenEzra D**, Cohen E, Chajek T, Friedman G, Pizanti S, de Courten C, Harris W. Evaluation of conventional therapy versus cyclosporine A in Behçet's syndrome. *Transplant Proc* 1988; **20**: 136-143
28 **Caspers-Velu LE**, Decaux G, Libert J. Cyclosporine in Behçet's disease resistant to conventional therapy. *Ann Ophthalmol* 1989; **21**: 111-116, 118
29 **Sanderson IR**. Chronic inflammatory bowel disease. *Clin Gastroenterol* 1986; **15**: 71-87
30 **Sawyer A**, Walker TM, Terry SI. Behçet's syndrome with ileal involvement--the beneficial effect of sulphasalazine. *West Indian Med J* 1978; **27**: 218-221
31 **Foster GR**. Behçet's colitis with oesophageal ulceration treated with sulphasalazine and cyclosporin. *J R Soc Med* 1988; **81**: 545-546
32 **Tolia** V, Abdullah A, Thirumoorthi MC, Chang CH. A case of Behcet's disease with intestinal involvement due to Crohn's disease. *Am J Gastroenterol* 1989; **84**: 322-325
33 **Rogé J**. [Behçet's syndrome and the digestive tract]. *J Mal Vasc* 1988; **13**: 235-239
34 **Gardiner R**, Stevenson GW. The colitides. *Radiol Clin North Am* 1982; **20**: 797-817
35 **Turkcapar N**, Toruner M, Soykan I, Aydintug OT, Cetinkaya H, Duzgun N, Ozden A, Duman M. The prevalence of extraintestinal manifestations and HLA association in patients with inflammatory bowel disease. *Rheumatol Int* 2006; **26**: 663-668
36 **Houman H**, Ben Dahmen F, Ben Ghorbel I, Chouaib S, Lamloum M, Kchir N, Ben Ammar A, Miled M. [Behçet's disease associated with Crohn's disease]. *Ann Med Interne* (Paris) 2001; **152**: 480-482
37 **Köksal AS**, Ertugrul I, Dişibeyaz S, Tola M, Kacar S, Arhan M, Aydin F, Ulker A. Crohn's and Behçet's disease association presenting with superior vena cava thrombosis. *Dig Dis Sci*

2005; **50**: 1698-1701
38 **Kallinowski B**, Nöldge G, Stiehl A. Crohn's disease with Behçet's syndrome like appearance: a case report. *Z Gastroenterol* 1994; **32**: 642-644
39 **Kobashigawa T**, Okamoto H, Kato J, Shindo H, Imamura T, Iizuka BE, Tanaka M, Uesato M, Ohta SJ, Terai C, Hara M, Kamatani N. Ulcerative colitis followed by the development of Behçet's disease. *Intern Med* 2004; **43**: 243-247
40 **Akay N**, Boyvat A, Heper AO, Soykan I, Arica IE, Bektas M, Ensari A, Cokca F. Behçet's disease-like presentation of bullous pyoderma gangrenosum associated with Crohn's disease. *Clin Exp Dermatol* 2006; **31**: 384-386

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World J Gastroenterol 2006 September 21; 12(35): 5753
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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
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International Gastrointestinal Fellows Initiative
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Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
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12th International Symposium on Viral Hepatitis and Liver Disease
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Falk Seminar: XI Gastroenterology Seminar Week
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European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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