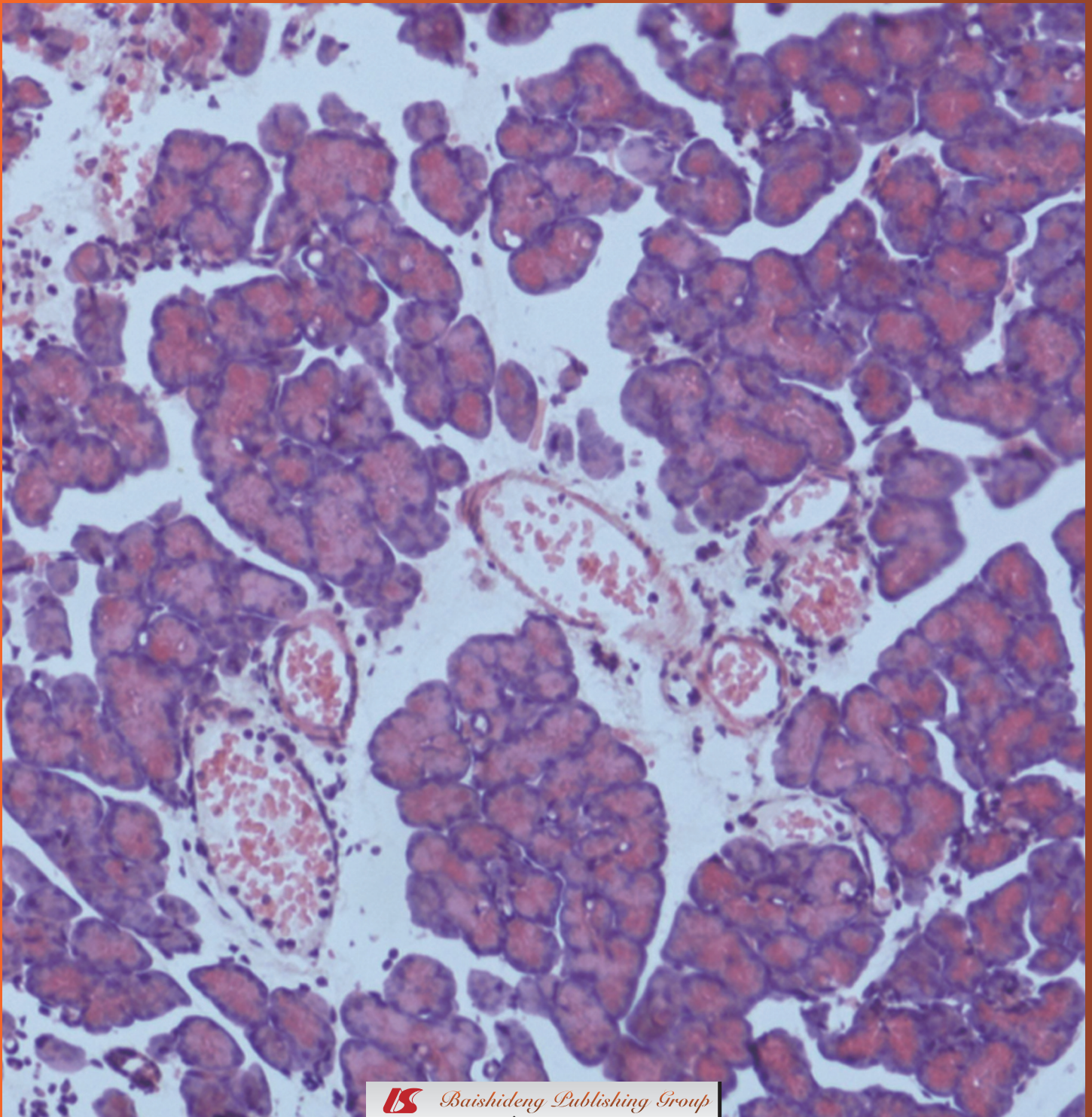


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Wound healing of intestinal epithelial cells

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wound healing, and the functions and mechanisms of the various factors that contribute to gut homeostasis and intestinal epithelial wound healing.

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Abstract

The intestinal epithelial cells (IECs) form a selective permeability barrier separating luminal content from underlying tissues. Upon injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the balance of three cellular events; restitution, proliferation, and differentiation of epithelial cells adjacent to the wounded area. Previous studies have shown that various regulatory peptides, including growth factors and cytokines, modulate intestinal epithelial wound healing. Recent studies have revealed that novel factors, which include toll-like receptors (TLRs), regulatory peptides, particular dietary factors, and some gastroprotective agents, also modulate intestinal epithelial wound repair. Among these factors, the activation of TLRs by commensal bacteria is suggested to play an essential role in the maintenance of gut homeostasis. Recent studies suggest that mutations and dysregulation of TLRs could be major contributing factors in the predisposition and perpetuation of inflammatory bowel disease. Additionally, studies have shown that specific signaling pathways are involved in IEC wound repair. In this review, we summarize the function of IECs, the process of intestinal epithelial

INTRODUCTION

The surface of the gastrointestinal tract is covered with epithelial cells that function under physiological conditions as a barrier preventing undesirable luminal antigens from entering the body^[1]. Upon injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the precise balance of migration, proliferation, and differentiation of the epithelial cells adjacent to the wounded area^[2]. Previous studies have shown that various regulatory peptides, including growth factors and cytokines, modulate intestinal epithelial wound healing^[3]. Recent studies have revealed that novel factors, which include toll-like receptors (TLRs)^[4], regulatory peptides^[5], particular dietary factors^[6,7], and some gastroprotective agents^[8-10], also modulate intestinal epithelial wound repair. In addition, it has been shown that the activation of specific signaling pathways is involved in intestinal epithelial wound healing^[11,12].

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory intestinal disorders and known as

inflammatory bowel disease (IBD). Although an etiology of both diseases remains unknown, it is suggested that disruption of the intestinal barrier function and repeated intestinal epithelial damage are key features of IBD, as well as other intestinal disorders^[1,3]. In this context, it is suggested that the identification of the specific factors that improve wound healing of the intestinal epithelium might contribute to therapeutic strategies in IBD, and many studies using animal colitis models and intestinal epithelial cell (IEC) lines have been conducted.

We summarize the function of intestinal epithelium, the process of intestinal epithelial wound healing, and the functions and mechanisms of the various factors that contribute to intestinal epithelial wound healing and gut homeostasis. We also discuss the association of some of these factors with IBD.

BARRIER FUNCTION OF INTESTINAL EPITHELIAL CELLS

The intestinal epithelial cells (IECs) form a selective permeability barrier separating luminal content from underlying tissues^[1,13]. The gastrointestinal epithelial lining consists of a monolayer of columnar cells^[13]. This monolayer of IECs is constantly moving at a speed of 5-10 $\mu\text{m}/\text{h}$ ^[14] and is renewed every 2-5 d. The maintenance of this barrier is critical for normal growth, development, and disease prevention^[15]. Normally, IECs function as a barrier that prevents undesirable solutes, microorganisms, viruses, and luminal antigens from entering the body^[1,16]. Several elements that participate in the barrier function include the epithelial cells themselves along with tight junctions, adherens junctions, and luminal secretions such as mucus or unstirred layers on the apical aspects of the epithelium^[1].

Tight junctions function as semi-permeable gates that regulate the passive movement of luminal fluid and solutes through the paracellular pathway, and limit passive diffusion of proteins and lipids between the outer leaflet of the apical and basolateral plasma membrane domains^[1,17]. Subjacent to tight junctions, adherens junctions are important in regulating intercellular adhesion^[1]. Both tight junctions and adherens junctions are positioned in the apical end of the lateral plasma membrane, and are intimately linked in their regulation and function. Thus, the tight junctions and adherens junctions are collectively referred to as the apical junctional complex (AJC)^[1,13,17]. Major transmembrane proteins in the AJC include occludin, claudins, junctional adhesion molecules (JAMs), coxsackie adenovirus receptor (CAR), and E-cadherin^[1]. Subjacent to the AJC are spot-like intercellular junctions referred to as desmosomes; the function of which in IECs is poorly understood^[13].

Despite the barrier function of IECs, this barrier has to allow some non-pathological gut-derived bacteria access to the immune system, thereby promoting maturation of the immune system and evolution of immune tolerance^[1]. As is described later, recent studies have

demonstrated that the activation of TLRs by commensal bacteria plays an essential role in inhibiting inflammatory responses and maintaining colonic homeostasis^[18].

PROCESS OF WOUND HEALING OF INTESTINAL EPITHELIAL CELLS

The intestinal epithelium can be injured by toxic luminal substances, normal digestion, inflammation, interactions with microbes, oxidative stress, and pharmaceuticals, despite its barrier function^[19,20]. After injury, the intestinal epithelium undergoes a wound healing process. Intestinal wound healing is dependent on the precise balance of migration, proliferation, and differentiation of the epithelial cells adjacent to the wounded area^[2]. First, epithelial cells surrounding the wound lose their columnar polarity, take on a flattened morphology, and rapidly migrate into the denuded area to restore barrier integrity. This process has been termed “epithelial restitution”^[3,15,21]. Restitution starts within minutes to hours of injury and is independent of proliferation^[3,21]. Then, proliferation of the mucosal epithelium to increase the pool of enterocytes available to resurface the defect generally begins hours or days after the injury^[21]. Finally, maturation and differentiation of epithelial cells is needed to maintain the mucosal barrier function^[3]. Rat IECs (IEC-6) at 24 h after wound formation are shown in Figure 1A. Most of the epithelial cells found in the wounded area across the wound border are thought to have migrated in the process of restitution. The process of repair of IEC-6 cells wounded by mechanical cell denudation is shown in Figure 1B. These wound assays were performed in our laboratory.

During the restitution of IECs, extensive reorganization of the actin cytoskeleton is necessary^[22]. The organization and remodeling of the actin cytoskeleton is suggested to be controlled by the Rho family of small GTPases, which includes Rho, Rac and Cdc42^[23]. These proteins have been implicated in the formation of stress fibers, lamellipodia (cytoskeletal protein actin projection on the mobile edge of the cell), and filopodia (slender cytoplasmic projections, similar to lamellipodia, which extend from the leading edge of migrating cells)^[23]. In detail, Rho regulates stress fibers and focal adhesion assembly; Rac regulates the formation of lamellipodia protrusions and membrane ruffles; and Cdc42 triggers filopodial extensions at the cell periphery^[24,25]. The wound margin of IEC-6 cells is shown in Figure 1C. Lamellipodia- and filopodia-like epithelial cells are found at the leading edge of the wounded IEC-6 cells (Figure 1C). Other investigations regarding the mechanism of cell migration have also been reported^[26-28].

Chemokines can be subdivided into distinct inflammatory or homeostatic subsets, with the latter being minimally regulated by pro-inflammatory cytokine stimulation^[29]. It has been shown in leukocytes that Rho-GTPase regulates homeostatic chemokine receptor CXCR4-mediated chemotaxis or metastasis to the sites of cognate

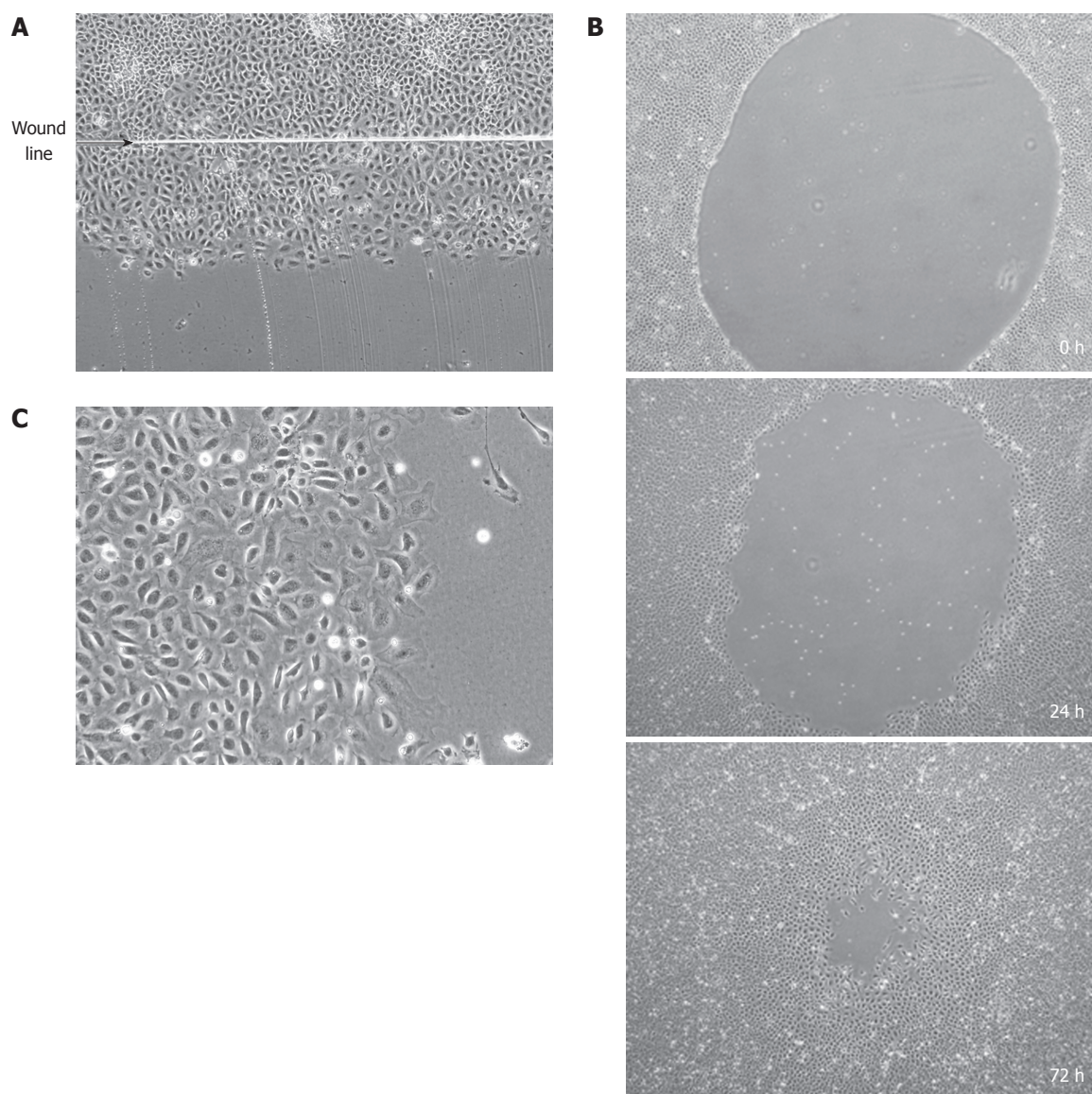


Figure 1 Wounded rat intestinal epithelial cell-6 cells. A: Confluent monolayers of intestinal epithelial cell (IEC)-6 cells were wounded with a razor blade. Wounded IEC-6 cells 24 h after wound formation. Wound line is shown with an arrow; B: IEC-6 cells wounded by mechanical cell denudation ($\times 40$). Wounded cells at 0, 24 and 72 h after wound formation are shown; C: Wound margin of IEC-6 cells ($\times 200$).

ligand CXCL12 production^[30]. Moyer *et al*^[2] have demonstrated that CXCL12 increases Rho-GTP and F-actin localization to the leading edge of wounded IEC-6 and T84 monolayers. Since Rho-GTP is known to be required for membrane protrusions in intestinal cell lines^[28], it has been postulated that CXCR4-mediated activation and localization of Rho at the leading edge of restitutive epithelial cells may facilitate the formation of lamellae that are required for wound healing^[2]. It has also been suggested that CXCR4 and CXCL12 function as an autocrine and paracrine mucosal signaling network that regulates the competency of the epithelial barrier to withstand injury and mediate repair following damage^[2].

Defensins are highly conserved key molecules that participate in host defense through the direct killing of microbes^[31]. Like the homeostatic chemokine receptor CXCR4, the chemokine receptor CCR6 is expressed by

immature dendritic cells and circulating T cells, which directs their trafficking to sites of inflammation following binding by the chemokine ligand CCL20^[19,32]. Vongsa *et al*^[19] have demonstrated that human β -defensins and CCL20 stimulated accumulation of F-actin, phosphorylation of the myosin light chain and RhoA, and restitutive migration of IEC-6 cells. These findings suggest that chemokines and β -defensins are protective host defense molecules that function not only to recruit immune cells and kill microbes, but also to increase the efficiency of wound healing in the gut. Annexin 2 is a calcium-dependent phospholipid-binding protein that also plays a role in regulating the actin cytoskeleton, and has been implicated in cell migration^[33,34]. It has been shown that annexin 2 regulates IEC migration and wound closure through Rho-dependent signaling pathways and related actin cytoskeletal remodeling^[33]. We have summarized various

Table 1 Various factors promoting or inhibiting wound healing of intestinal epithelium and mucosa

Categories	Factors	Promoting effect	Inhibitory effect	No effect	Models
(Chemokines)	CXCL12	+			Cells ^[2]
	CCL20, defensins	+			Cells ^[19]
Annexin 2		+			Cells ^[33]
(Growth factors)	TGF- α	+			Cells ^[21,44]
	TGF- β	+			Cells ^[40]
	EGF, HB-EGF	+			Cells ^[11,21]
	HGF	+			Cells ^[36]
	FGF	+			Cells ^[35]
	KGF	+			Cells ^[35] , rat ^[37]
	IGF- I , - II	+			Mice ^[38] , cells ^[39]
(Cytokines)	IL-1 β	+			Cells ^[21]
	IL-2	+			Cells ^[41]
	IFN- γ	+ ^[21]	+ ^[5]		Cells ^[21] , mice and cells ^[5]
	PDGF, IL-6, TNF- α			+	Cells ^[21]
Trefoil peptide		+			Cells ^[42]
Prostaglandin, COX-1, COX-2		+			Mice ^[51-54]
(Toll-like receptors)	TLR2	+			Mice and cells ^[92-94,97]
	TLR3	+ ^[95]	+ ^[96]		Mice ^[95] , cells ^[96]
	TLR4	+ ^[97-99]	+ ^[100]		Mice ^[97-99] and cells ^[100]
	TLR5	+			Mice and cells ^[101,102]
	TLR9	+			Mice ^[103-105]
(Dietary factors)	Glutamine	+			Rats ^[6] , cells ^[68]
	Histidine	+			Mice ^[7]
	Vit D	+			Mice and cells ^[72]
(Gastroprotective agents)	Rebamipide	+			Rats ^[8,77]
	Ecabet sodium	+			Cells ^[9] and rats ^[79]
	Sucralfate	+			Cells ^[10]
(Other factors)	Epimorphin	+			Cells ^[60]
	Muc3	+			Mice and cells ^[62]
	HIF	+			Mice ^[63]
	GM-CSF	+			Mice ^[64]

COX: Cyclooxygenase; TGF: Transforming growth factor; EGF: Epidermal growth factor; HB-EGF: Heparin-binding epidermal growth factor; HGF: Hepatocyte growth factor; FGF: Fibroblast growth factor; KGF: Keratinocyte growth factor; IGF: Insulin-like growth factor; IL: Interleukin; IFN: Interferon; PDGF: Platelet-derived growth factor; TLR: Toll-like receptor; HIF: Hypoxia-inducible factor; GM-CSF: Granulocyte-macrophage colony stimulating factor; TNF: Tumor necrosis factor.

factors that can have specific effects on IEC or intestinal mucosal wound healing in Table 1.

REGULATORY PEPTIDES AND SIGNALING PATHWAYS IN INTESTINAL WOUND HEALING

Previous studies have shown that various growth factors [including transforming growth factor (TGF)- α and TGF- β , epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), insulin-like growth factor (IGF)- I and IGF- II], the cytokine interleukin (IL)-1 β and IL-2, and trefoil peptides enhance restitution or proliferation of IECs^[3,21,35-42]. In contrast, platelet-derived growth factor (PDGF), IL-6, and tumor necrosis factor (TNF)- α have no effect on cell migration^[21]. With regard to interferon (IFN)- γ , a previous study has shown that IFN- γ enhanced epithelial cell restitution by 3.8-fold^[21]. However, a recent study has shown that IFN- γ inhibits enterocyte migration by preventing inter-enterocyte gap junction communication^[5]. Among the regulatory peptides, TGF- β is known to be a central factor that intrinsically contributes to the restitution of wounded

IECs, although it inhibits IEC proliferation^[21]. It is noteworthy that cytokines (IL-1 β , IL-2), EGF, FGF, HGF and TGF- α promote epithelial cell restitution through enhanced production of bioactive TGF- β , namely, the TGF- β -dependent pathway^[21,35,36,41]. On the other hand, it has been shown that both trefoil peptides, which are secreted onto the surface of the gastrointestinal tract, and galectin-2 and -4, which ameliorate colitis in several models of intestinal inflammation, promote IEC restitution through a TGF- β independent pathway^[42,43]. In contrast, TGF- α mediates its stimulatory effects on the proliferation and restitution of IECs through different mechanisms; as described above, it enhances IEC restitution through a TGF- β -dependent pathway but promotes IEC proliferation through the activation of extracellular signal-regulated kinase (ERK)1/ERK2 mitogen-activated protein kinase (MAPK)^[44].

It has been shown that the activation of specific signaling pathways is involved in intestinal epithelial wound repair. El-Assal *et al.*^[11] have demonstrated that heparin-binding epidermal growth factor-like growth factor (HB-EGF) enhances restitution of the intestine *in vivo* and *in vitro* in a phosphatidylinositol 3-kinase (PI3K)/Akt- and MAPK/ERK kinase (MEK)/ERK1/2-dependent fashion. Using mouse epithelial cell lines, Dise *et al.*^[15] have

shown that the PI3K and Src signaling cascades cooperate with Rac and promote IEC migration in response to EGF. Other studies have shown that the activation of the ERK1/2 MAPK or PI3/Akt pathway plays an important role in the regulation of intestinal epithelial proliferation, survival, and wound healing^[12,45-47]. Recent studies have suggested that nuclear factor (NF)- κ B has not only pro-inflammatory but also has a tissue-protective function in IECs^[48]. In addition, Pickert *et al.*^[49] have reported using conditional knockout mice with an IEC-specific deletion of signal transducer and activator of transcription (STAT) 3 activity that intestinal epithelial STAT3 activation regulates immune homeostasis in the gut by promoting IL-22 dependent mucosal wound healing. Trem2 is a cell surface receptor that is specifically induced in macrophages by IL-4/IL-13. Seno *et al.*^[50] have shown that Trem2 signaling promotes efficient wound healing of colonic mucosal injuries by inhibiting cytokines that can enhance M1 macrophage activation, and by promoting cytokines that can promote M2 macrophage activation.

Previous studies have shown that prostaglandin (PG) E2 plays a major role in the regeneration of the epithelial crypts and in the prevention of decreased epithelial cells proliferation after radiation- or dextran sodium sulfate (DSS)-induced intestinal injury^[51,52]. Mucosal PGs are synthesized from arachidonate by cyclooxygenase (COX)-1 or COX-2^[52]. Thus, it has also been shown that COX-1 and COX-2 share a crucial role in the defense of the intestinal mucosa^[53,54]. In this context, Brun *et al.*^[55] have shown that the neuropeptide neurotensin (NT) significantly increases COX-2 mRNA levels and stimulates PGE2 release in the colonic cell line HT29. They also have shown that NT significantly enhances the migration of HT-29 cells into the denuded area of a wound model. It has also been shown that PGE2 reduces radiation-induced apoptosis in the intestine through transactivation of the EGFR, enhanced activation of Akt, and reduced Bax translocation from the cytoplasm to the mitochondria^[56].

Epithelial-mesenchymal interactions are necessary for proper gut morphogenesis^[57]. These interactions also play important roles in intestinal epithelial wound healing. Epimorphin is expressed on the surface of mesenchymal cells in various organs, including intestinal mucosa, and is suggested to play a key role in the morphogenesis of epithelial cells^[58,59]. We found that epimorphin also has a novel function to promote restitution of IECs under oxidative stress conditions through the activation of the EGF receptor and MEK/ERK, PI3K/Akt signals^[60]. HGF, FGF and KGF are also secreted by mesenchymal cells^[57]. Göke *et al.*^[61] have shown that fibroblasts, which are derived from primitive mesenchyme, promote intestinal cell proliferation in addition to affecting secretory responses, differentiation, and morphogenesis, and that this function is predominantly mediated by the paracrine action of HGF.

Recent studies using experimental colitis models have shown that the Muc3 mucin cysteine-rich domain^[62], hypoxia-inducible factor (HIF)^[63], and granulocyte-mac-

rophage colony stimulating factor (GM-CSF)^[64] improve wound healing of the colonic mucosa. We summarize the various factors and signaling pathways that contribute to intestinal epithelial and mucosal wound healing in Figure 2.

DIETARY FACTORS AND INTESTINAL WOUND HEALING

Several studies have shown that enteral nutrition with an elemental diet is efficacious in the treatment of CD, especially for maintaining clinical remission or reducing clinical and endoscopic recurrence after resection^[65-67]. Although some of this effectiveness may be due to the low antigenic load, low fat content, and modulation of the commensal bacterial flow, the precise mechanisms remain unclear. In this context, the effectiveness of the constituent amino acids of the elemental diet for intestinal epithelial and mucosal wound healing has been reported.

Glutamine, the most abundant free amino acid in the bloodstream, is a non-essential amino acid that is essential for gut homeostasis and is an essential respiratory substrate for cells in the small intestinal mucosa^[68,69]. Enteral glutamine is thought to stimulate intestinal mucosal protein synthesis and protect against apoptosis^[68]. Larson *et al.*^[68] have shown that glutamine supplementation stimulates IEC growth and prevents apoptosis, and that activation of ERK is an important contributor to glutamine-mediated intestinal cell survival. Sukhotnik *et al.*^[6] have evaluated the preventive effects of oral glutamine supplementation in an intestinal ischemia-reperfusion injury in a rat. It was found that pretreatment with oral glutamine prevents mucosal injury and improves intestinal recovery following ischemia-reperfusion injury through the stimulation of cell proliferation rather than the inhibition of cell apoptosis.

On the other hand, Andou *et al.*^[7] have assessed the role of histidine, an essential amino acid, in controlling colitis by using an IL-10-deficient cell transfer model. In this study, it was shown that dietary histidine reduced histological damage of the colon and TNF- α mRNA expression by inhibiting NF- κ B activation, following the down-regulation of pro-inflammatory cytokine production by macrophages. Son *et al.*^[70] also have reported that histidine significantly inhibits both hydrogen peroxide- and TNF- α -induced IL-8 secretion and mRNA expression in intestinal epithelial-like cell lines. This study has also shown that histidine abolishes the NF- κ B-dependent activation of the IL-8 promoter induced by TNF- α , suggesting that histidine has the potential to attenuate intestinal inflammation. These reports investigating the function of glutamine and histidine have suggested the efficacy of an elemental diet for improvement of intestinal mucosal wound healing in patients with CD.

Previous studies have suggested a link between vitamin D deficiency and IBD risk^[71]. Kong *et al.*^[72] have investigated the role of the vitamin D receptor (VDR) in mucosal barrier homeostasis by using the DSS-induced colitis model. In this study VDR^{+/-} mice were mostly resistant

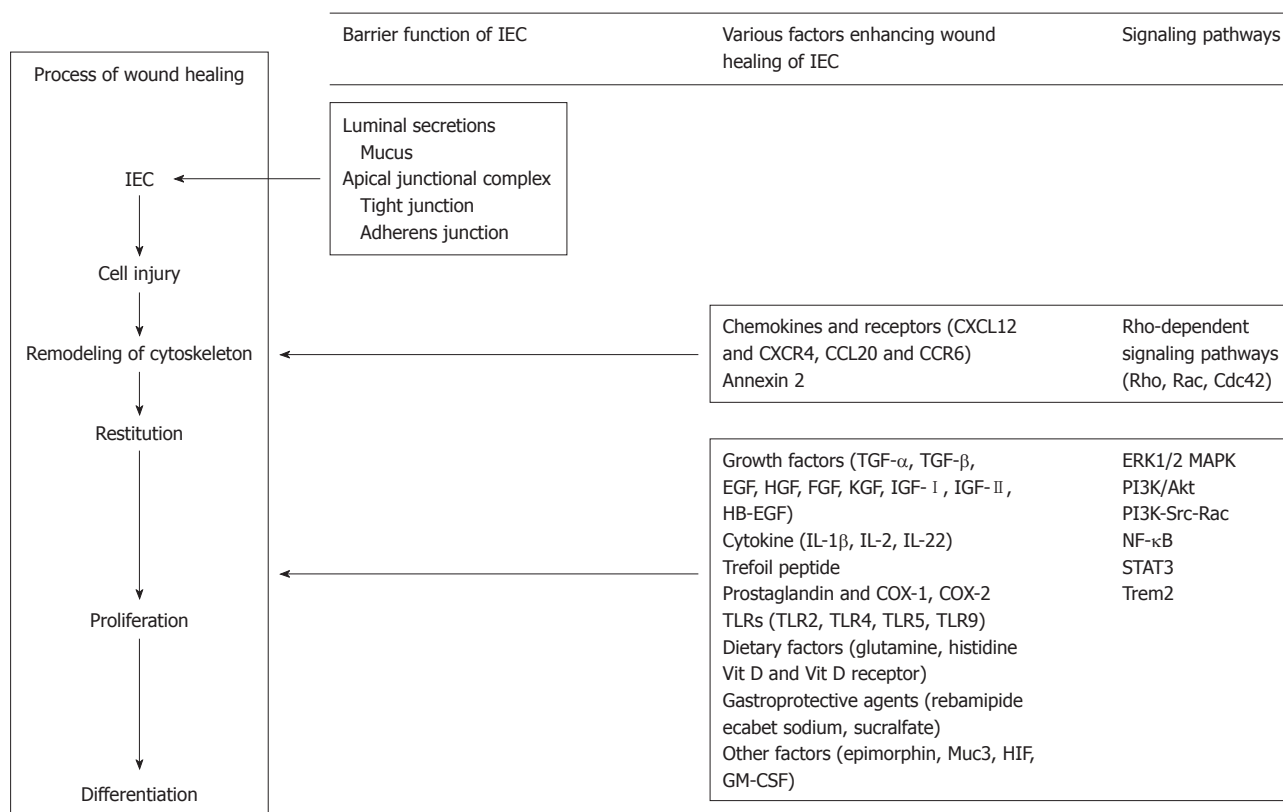


Figure 2 Various factors and signaling pathways contributing to the process of wound healing of intestinal epithelial cells and intestinal mucosa. IEC: Intestinal epithelial cell; TGF: Transforming growth factor; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor; FGF: Fibroblast growth factor; KGF: Keratinocyte growth factor; IGF: Insulin-like growth factor; HB-EGF: Heparin-binding epidermal growth factor; IL: Interleukin; COX: Cyclooxygenase; TLR: Toll-like receptor; HIF: Hypoxia-inducible factor; GM-CSF: Granulocyte-macrophage colony stimulating factor; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; NF: Nuclear factor; STAT: Signal transducer and activator of transcription.

to 2.5% DSS, but VDR^{-/-} mice developed severe colitis, leading to death. They also found severe disruption in the epithelial junctions in VDR^{-/-} mice after DSS treatment. In cell cultures, 1,25-dihydroxy-vitamin D₃ [1,25(OH)₂D₃] markedly enhanced tight junctions and stimulated epithelial cell migration *in vitro*. These observations suggest that VDR plays a critical role in mucosal barrier homeostasis by preserving the integrity of junction complexes and the healing capacity of the colonic epithelium.

GASTROPROTECTIVE AGENTS IN INTESTINAL EPITHELIAL WOUND HEALING

Recent studies have shown that some gastroprotective agents, including ecabet sodium (ES), rebamipide, and sucralfate, have therapeutic effects on IBD and other types of colitis^[73-76]. Previous studies have shown that rebamipide enema is effective for treatment of experimental DSS-induced colitis in rats^[8,77]. Okayama *et al*^[77] have suggested that the protective effect of rebamipide may be attributable to both the radical scavenging action and the increase in the production of mucus in the colon. Watanabe *et al*^[78] have performed wound assays using gastric epithelial cells and have shown that rebamipide

prevents delay of wound repair induced by hydrogen peroxide. On the other hand, Sasaki *et al*^[9] have investigated the therapeutic mechanism of ES for intestinal mucosal injury by using the rat IEC-6 cell line. The investigation clarified that ES prevents the delay of wound repair in IEC-6 cells induced by hydrogen peroxide, probably through the activation of ERK 1/2 MAPK and the induction of COX-2. Takagi *et al*^[79] have shown that intra-colonic administration of ES accelerates TNBS-induced ulcer healing *in vivo*. Shindo *et al*^[10] have investigated the therapeutic mechanism of sucralfate for intestinal mucosal injury. It has been demonstrated that sucralfate also prevents the delay of wound repair in IEC-6 cells induced by hydrogen peroxide through the induction of COX-2 and an anti-apoptotic mechanism. The effects of sucralfate may be initiated by the activation of the NF- κ B pathway. These studies have suggested the possibility that some gastroprotective agents could be used for the treatment of IBD.

ROLE OF THE INNATE IMMUNE SYSTEM IN COLONIC HOMEOSTASIS AND INTESTINAL WOUND HEALING

The commensal bacteria population is comprised of at

least 400 species, with a load of as many as 10^{12} bacteria per gram of intestinal content^[80]. As described previously, IECs are the first line of defense against commensal or pathogenic luminal microflora. Recognition of enteric bacteria is mediated by several mechanisms, the most important of which rely on host receptors specific for conserved bacterial structures not found in the host^[81]. The two major host receptors currently recognized in humans are TLRs and the nucleotide-binding oligomerization domain (NOD)-containing proteins^[81]. In this context, it is notable that mutations in NOD2, a cytoplasmic innate immune-recognition receptor, are associated with susceptibility to CD^[82,83]. TLRs are innate immune-recognition receptors that bind a spectrum of pathogen-associated molecular patterns (PAMPs) present in pathogenic and commensal bacteria and viruses, as well as some endogenous proteins^[84-86]. TLR family members were first noted to be expressed by immune cells such as monocytes and dendritic cells^[84-86], then, it was shown that IECs also express TLRs^[4]. With regard to the association of TLRs with IBD, several studies have shown the alteration of TLR expression and the specific antibody to the TLR ligand in patients with IBD. First, Cario *et al.*^[87] reported the upregulation of TLR4 in IECs in UC and CD, and the downregulation of TLR3 in CD. A recent study has shown the association between the TLR2 polymorphism R753Q and the severe UC phenotype^[88]. Such associations have also been found between the TLR4 polymorphism at Asp299Gly and the development of CD and UC, and the TLR4 polymorphism at Thr399Ile and UC^[89,90]. On the other hand, the presence of high titers of flagellin-specific antibodies in the serum of CD patients has been reported^[91].

The following *in vivo* and *in vitro* studies have provided evidence that each TLR contributes to intestinal homeostasis and intestinal mucosal repair. TLR2 recognizes lipoproteins derived from a variety of bacteria, peptidoglycan, lipoteichoic acid from many Gram-positive bacteria, and lipopolysaccharide (LPS) from *Leptospira* and *Porphyromonas gingivalis*^[4]. Cario *et al.*^[92] have shown that TLR2 stimulation effectively preserves the tight junction-associated barrier assembly in IECs against stress-induced damage, and suppresses mucosal inflammation and apoptosis of IECs *in vivo*. The same group has shown that TLR2 controls gap junction intercellular communication and commensal-mediated intestinal epithelial wound repair by modulating intestinal epithelial connexin-43^[93]. Furthermore, they have shown that TLR2 controls terminal goblet cell differentiation by selectively regulating trefoil factor 3 expression in the intestine and confers anti-apoptotic protection to the intestinal mucosa^[94].

TLR3 recognizes double-stranded RNA, which is a molecular pattern associated with viral infection^[86]. A recent study has shown that administration of TLR3 ligand polyinosinic acid: cytidylic acid protects against DSS-induced colitis^[95]. In contrast, Sato *et al.*^[96] have performed

wound assays using IEC-6 cells and have shown that TLR3 ligand rotavirus double-stranded RNA induces apoptosis and diminishes wound repair of IECs *in vitro*.

TLR4 recognizes LPS that is an integral component of the outer membranes of Gram-negative bacteria^[4]. Using DSS-induced colitis mice, Rakoff-Nahoum *et al.*^[97] have demonstrated that commensal bacteria are recognized by TLRs (TLR2 and TLR4) under normal steady-state conditions, and that this interaction plays a crucial role in the maintenance of intestinal epithelial homeostasis. They have shown that activation of TLRs by either the TLR2 or TLR4 ligand is critical for the protection against gut injury and associated mortality. This group has also shown that TLR-mediated signaling plays a critical role in intestinal inflammation in the context of deficiency in the anti-inflammatory cytokine IL-10, but not in the context of insufficient activity of regulatory T cells^[98]. Another study using a murine colitis model also has shown that TLR4 is important in intestinal response to injury and in limiting bacterial translocation^[99]. On the other hand, Cetin *et al.*^[100] have reported that LPS inhibits IEC-6 cell migration through a RhoA-dependent increase in focal adhesions and enhanced cell adhesiveness.

Flagellin, the primary structural component of bacterial flagella, is recognized by TLR5 present on the basolateral surface of IECs^[101]. A recent study has shown that, in salmonella infections, flagellin plays a dominant role in the activation of not only innate immunity but also anti-apoptotic processes in epithelial cells^[102]. The same group also has shown that pretreatment of epithelial cells with flagellin can protect cells from a subsequent bacterium-mediated apoptotic challenge through the activation of NF- κ B and PI3K/Akt signaling^[101]. These studies suggest that the TLR5 ligand flagellin has a fundamental cytoprotective effect in inflammatory stress.

TLR9, an intracellular protein in immune cells, is expressed on the surfaces of IECs, both on the apical and basolateral membrane^[18]. TLR9 recognizes CpG motifs, derived from bacterial DNA^[4]. Recent studies have demonstrated that TLR-9 signaling in IECs contributes to colonic homeostasis^[18,103-105]. Katakura *et al.*^[103] have shown that TLR9 agonist suppresses the severity of experimental colitis by inducing type I IFN (IFN- α/β). The same group also has shown that administration of probiotics (non-viable irradiated or viable probiotics) ameliorates the severity of DSS-induced colitis^[105]. They have suggested that TLR9 signaling is essential in mediating the anti-inflammatory effect of probiotics, and that live microorganisms are not required to attenuate experimental colitis. They have shown that while basolateral TLR9 signaling is fully capable of inducing an NF- κ B-mediated pro-inflammatory response, apical TLR9 signaling does not induce an inflammatory response due to a defect in NF- κ B activation^[18,104]. They have suggested that stimulation of apical TLR9 compromises the inflammatory cascade induced basolaterally by several other TLR ligands, and thus, apical exposure to luminal microbial DNA restrains

intestinal inflammation and supports colonic homeostasis *via* the activation of TLR9.

In conclusion, most of these studies investigating the function of TLRs strongly suggest the possibility that the activation of TLRs by commensal bacteria plays an essential role in maintaining colonic homeostasis and protection of IECs from gut injury. These studies also support the therapeutic efficacy of some antibiotics^[106-108] or probiotics^[109] in IBD patients. Thus, modulations of host-commensal interactions *via* TLRs might be targeted for optimal therapeutic strategies in IBD.

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Mallory-Denk Bodies in chronic hepatitis

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Abstract

Mallory-Denk Bodies (MDB) are important as investigators, suggesting MDB as an indicator of the histologic severity of chronic hepatitis, causes of which include hepatitis C, primary biliary cirrhosis (PBC), and nonalcoholic fatty liver disease (NAFLD). Matteoni *et al* scored MDB in patients with NAFLD as none, rare and many, and reported that MDB plays a prominent role in this classification scheme in an earlier classification system. In this study, we evaluated 258 patients with chronic hepatitis due to metabolic, autoimmune and viral etiologies. Liver biopsy samples were evaluated with hematoxylin and eosin, periodic acid-Schiff-diastase, Gordon and Sweet's reticulin, Masson's trichrome, and iron stains. Both staging and grading were performed. Additionally, MDB were evaluated and discussed for each disease. We examined patients with nonalcoholic steatohepatitis (NASH; 50 patients), alcoholic hepatitis (10 patients), PBC (50 patients), Wilson disease (WD; 20 patients), hepatitis B (50 patients), hepatitis C (50 patients)

and hepatocellular carcinoma (HCC; 30 patients). Frequency of MDB was as follows; NASH: 10 patients with mild in 60% and moderate in 40% and observed in every stage of the disease and frequently seen in zone 3. PBC: 11 patients with mild in 10%, moderate in 70%, and cirrhosis in 20%, and frequently seen in zone 1. WD: 16 patients with moderate and severe in 60% and cirrhosis in 40% and frequently seen in zone 1. Hep B: 3 patients with mild in 66% and severe in 34%. Hep C: 7 patients with mild in 40% and moderate in 60% and observed in every stage. HCC: 3 patients with hep B in 2 patients. We found that there is no relationship between MDB and any form of chronic hepatitis regarding histologic severity such as alcoholic steatohepatitis and NAFLD and variable zone distribution by etiology.

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Key words: Non-alcoholic fatty liver disease; Mallory-Denk Bodies; Hepatitis B and C; Hepatocellular carcinoma; Primary biliary cirrhosis; Wilson disease

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HISTORICAL BACKGROUND OF MALLORY-DENK BODIES IN HEPATOLOGY

Frank Burr Mallory first reported Mallory-Denk Bodies (MDB) in patients with alcoholic cirrhosis in 1911^[1]. Then, this pearl of pathology was reported in various hepatic diseases such as the hepatitis B and C virus, Wilson's

disease, chronic cholestatic injuries, nonalcoholic fatty liver disease (NAFLD), drug injuries, focal nodular hyperplasia, and hepatocellular carcinoma (HCC).

THE PATHOGENESIS OF MDB FORMATION IN CHRONIC HEPATITIS: WHAT WE KNOW

MDB are an intracellular deposition of misfolded protein aggregated into ubiquitin (Ub)-rich cytoplasmic inclusions in ballooned hepatocytes^[2]. MDB formation, which consists of abnormally phosphorylated, ubiquitinated, and cross-linked keratins and non-keratin components, are not entirely interchangeable since not all ballooned hepatocytes contain MDB. Both ballooning of hepatocytes and MDB are the two hallmarks of ongoing inflammation (Figure 1). To understand the pathogenesis of MDB, we first have to know the development of ballooning of hepatocytes.

Ballooning or swelling of hepatocytes is induced by oxidative stress and its products such as oxyradicals. The swelling of hepatocytes could be explained by water accumulation in the cytoplasm as a response to accumulated stress proteins such as heat shock proteins (HSPs) or fat. HSPs are the precursor of MDB and indicate hepatocytes injury^[2,3]. Currently, we do not know exactly whether ballooning are adaptive (physiological, reversible) or degenerative changes (pathological, most likely irreversible) of hepatocytes against the changed environment.

Swelling of hepatocytes represents volume increase (hydration) of the hepatocytes. It occurs against different stressors, particularly oxidative stress. It is reported that mild volume changes (up to 10% increase on the volume of the hepatocytes) without the biochemical evidence of free radicals are physiologic and adaptive. Hepatocyte damage may not be observed. However, high grade swelling (such as at least 30% increase on the volume of the hepatocytes) could be degenerative and can cause hepatocyte apoptosis, necrosis, and even death.

Most of the previous studies came from animal models. A hepatocyte includes two big compartments, namely wet (water) and cellular dry solids (fat, protein, nucleic acids, anions and cations and other solutes) which are in balance. Any intracellular solute (such as fat or abnormal proteins) accumulation into the cytoplasm may cause water movement, and water volume increase within the hepatocyte. This may change hepatocyte membrane transport system activation (such as ion channels) and may increase intracellular metabolism (increase both protein and glycogen synthesis and inhibit both proteolysis and glycogenolysis). The only purpose of these mechanisms is to maintain the functioning of both hepatocytes, and the liver as an organ. The volume ratio between these two compartments should be maintained at the same level under normal conditions and also against stressors.

It is experimentally shown in rats that the initial response of hepatocytes against iron, which is a well-known strong oxidative agent, was the accumulation of increased

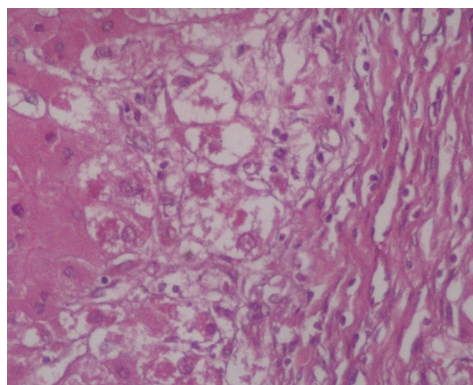


Figure 1 Zone 1 distribution of Mallory-Denk Bodies in primary biliary cirrhosis ($\times 400$). This distribution was observed in both early and later stages of primary biliary cirrhosis, such as cirrhosis.

stress proteins, such as HSPs, within the cytoplasm. Increased stress proteins, along with the other elements of the increased metabolic process, cause macromolecular crowding and activate volume regulator mechanisms. To maintain the ratio between wet (water) and increased dry compartments (such as HSPs), volume regulator mechanisms increase the hepatocyte hydration with increasing water content of the cytoplasm (cloudy swelling). The real mission of this regulatory system is to preserve the intracellular environment and hepatocyte functioning. This is an adaptation mechanism of hepatocytes against oxidative stress (up to 10% increase on the volume of the hepatocytes). However, too much increased water within the cytoplasm of hepatocytes may cause degenerative changes and disturbances in both normal hepatocyte morphology and functioning. Then, hepatocyte apoptosis, necrosis and death may occur.

Additionally, we can discuss the toxic fatty liver animal model. Investigators used rats treated with CCl₄, which is a well-known steatogenic oxidant agent. CCl₄ is a cause of high grade hepatocyte swelling. This chemical poison was injected into the rats daily (one injection per day). Then the livers of the rats were examined for three compartments (water, fat, and fat-free dry solids) at the entry, after 1 injection, and after 6 injections. Investigators observed that both fat and water were increased significantly while fat-free dry solids were not changed or only slightly changed. The highest grade increase of water was seen after the sixth injection (25% water increase after 1 injection, and 48% water increase after 6 injections) which was related to the increased hepatocyte hydration. This means that there were many well-established ballooned hepatocytes in the liver of CCl₄ injected rats.

However, these protection mechanisms are not infinite. A strong oxidative stress may cause high amplitude ballooning of hepatocyte due to both increased hydration and dry solids. One of the increased cellular dry solids is stress protein, such as HSPs. HSPs, as examples of misfolded proteins, are the indicators of cellular dysfunction. Under normal conditions, these potentially harmful proteins are targeted by covalent attachment of multi-Ub

Table 1 Analysis of our 258 patients for Mallory-Denk Bodies and staging of the disease *n* (%)

	No. of pts	MDB (+) pts	Staging ¹ in pts with MDB	Comment
NASH	50	10 (20)	Mild in 60% and moderate in 40%	MDB observed in every stage of NASH, predominantly in zone 3
Alcoholic (ASH)	10	5 (50)	Mild in 40% and moderate in 60%	MDB observed in every stage, predominantly in zone 3
PBC	50	11 (22)	Mild in 10%, moderate in 70%, and cirrhosis in 20%	Frequently seen in later stages with predominantly in zone 1
WD	20	16 (80)	Moderate and severe in 60% and cirrhosis in 40%	Frequently seen in later stages with predominantly in zone 1
Hep B	48	3 (6)	Mild in 66% and severe in 34%	Mild case with steatosis
Hep C	50	7 (14)	Mild in 40% and moderate in 60%	MDB observed in every stage
HCC	30	3 (10)		Two cases with Hep B
Total	258	55 (21)		

¹Mild: Stage 1; Moderate: Stage 2-3; Severe: Stage 4, and cirrhosis. MDB: Mallory-Denk Bodies; ASH: Alcoholic steatohepatitis; NASH: Nonalcoholic steatohepatitis; PBC: Primary biliary cirrhosis; WD: Wilson disease; HCC: Hepatocellular carcinoma; pts: Patients.

chains. Then, a protective mechanism, the Ub-proteasome pathway eliminates these products. When this control system fails, under the strong oxidative stress, abnormal cytokeratins (CKs) may become accumulated along with HSPs (70, 90, and 25), Ub, tissue transglutaminase, proteasome subunits, tubulin, and p62. Young, tiny and then well-formed MDB were well established in ballooned hepatocytes. This mechanism has not been fully understood yet. There are two possible ways for this pathway to fail: (1) production of these misfolded proteins exceeding the capacity of this protective system or (2) inhibition of the pathway. Well-established MDB might be a degenerative rather than adaptive response of hepatocytes against stronger stressors. Then, satellite cell activation occurs. Sustained liver injury leads to fibrosis and cirrhosis.

In conclusion, there is no contrast of MDB pathogenesis in hep B, hep C, PBC, NAFLD and the others.

DIFFERENCES IN THE FREQUENCY AND ZONAL DISTRIBUTION OF MDB IN PATIENTS WITH CHRONIC HEPATITIS

MDB are typical features of alcoholic steatohepatitis (ASH) and NAFLD. NAFLD exhibits slightly less prominent MDB than ASH^[4]. MDBs can also be detected after intestinal bypass surgery for morbid obesity, in chronic cholestasis, PBS, Wilson disease (WD) and other types of copper toxicosis, various metabolic disturbances, and hepatocellular neoplasms. In idiopathic copper toxicosis and HCC, MDBs may coincide with another type of cytoplasmic inclusions.

MDB is not present in the majority of individuals with hep C, or even in hep C patients with NAFLD (the presence of MDB in patients with hep C is 7.1%). However, hepatocyte ballooning with MDB is much more likely to lead to advanced fibrosis in these patients.

MDB in periportal liver cells have been reported in patients with Wilson's disease. MDBs are found in mice by feeding them the hepatotoxic substances griseofulvin and 3,5-diethoxycarbonyl- 1,4-dihydrocollidine.

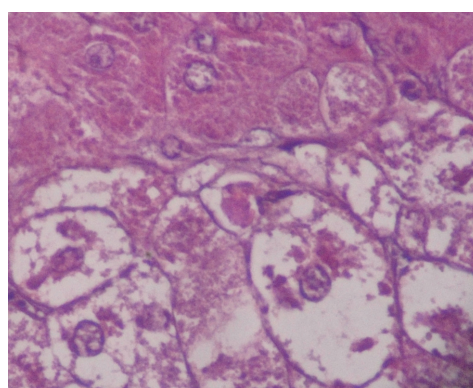


Figure 2 Mallory-Denk Bodies, located perinuclear, has been shown as a larger picture in a patient with primary biliary cirrhosis (× 1000).

MDBs have not been observed in acute cholestasis, acute viral hepatitis, acute toxic or drug-induced liver diseases.

OUR RESULTS: FREQUENCY OF MDB IN DIFFERENT FORMS OF CHRONIC HEPATITIC DISEASES

Two hundred and fifty-eight patients with hepatitis have been examined at our pathology department. Outpatient percutaneous percussion guided needle liver biopsies were performed using Menghini soft tissue biopsy needles (1.4 or 1.6 mm, adult size, Hepafix, Braun, Melsungen, Germany). Liver biopsy samples were evaluated with hematoxylin and eosin, periodic acid-Schiff-diastase, Gordon and Sweet's reticulin, Masson's trichrome, and iron stains by a single pathologist, unaware of the clinical and biochemical data. We examined patients with nonalcoholic steatohepatitis (NASH; 50 patients), alcoholic hepatitis (10 patients), primary biliary cirrhosis (PBC; 50 patients), WD (20 patients), hepatitis B (50 patients) and hepatitis C (50 patients), and HCC (30 patients) for grading and staging and MDB. Results were shown at Table 1. Furthermore, the characteristics of MDB in each disease have been

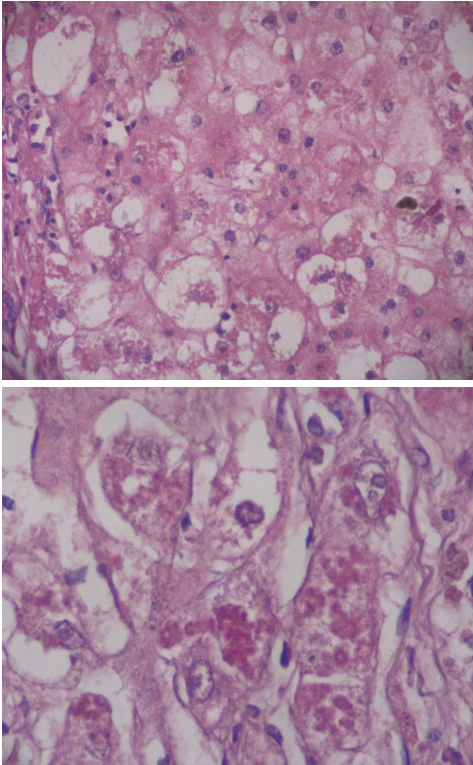


Figure 3 Mallory-Denk Bodies, located both perinuclear and intracytoplasmic, was observed in zone 1 in early stages of Wilson disease, but in every zone at the later stages, such as cirrhosis ($\times 400$, 1000 , respectively). Hydrophobic degeneration was more exaggerated. The diameter of Mallory-Denk Bodies was not homogenous in patients with Wilson disease.

shown in Figures 1-5. MDB showed zone 1 distribution in PBC and its distribution, located perinuclear, observed in both early and later stages, such as cirrhosis. MDB, located both perinuclear and intracytoplasmic, was observed in zone 1 in early stages of WD, but in every zone at the later stages, such as cirrhosis. Hydrophobic degeneration was more exaggerated in WD and the diameter of MDB was not homogenous in patients with WD. Perinuclear localized MDB in patients with HCC depends on hepatitis B, which is responsible for MDB. Lastly, tiny and uniform distribution of MDB was seen in patients with NASH. Although patients with PBC and WD showed MDB frequently in later stages, we found that there is no relationship between MDB and any form of chronic hepatitis regarding histologic severity, such as in ASH and NAFLD. Moreover, zone distribution of MDB is very variable by etiology, such as zone 1 in PBC and WD as zone 3 in ASH and NAFLD. Of the 3 hepatitis B patients with MDB, 2 showed steatosis with mild fibrosis. Most probably, MDB in these cases was caused by steatosis due to metabolic reasons.

MDB IN NAFLD

In an earlier classification system, Matteoni *et al*^[5] scored MDB in patients with NAFLD as none, rare and many. MDB plays a prominent role in this classification scheme as well as follows; Type 1: Fatty liver alone, Type 2: Fat

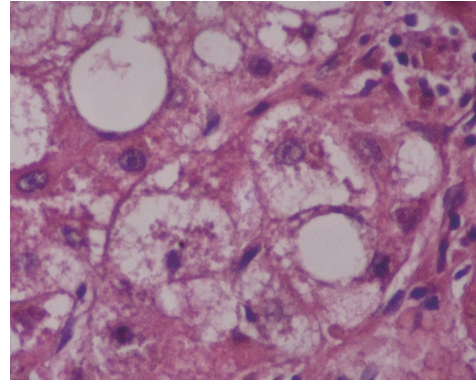


Figure 4 Perinuclear location of Mallory-Denk Bodies in patients with hepatocellular carcinoma depends on the cause ($\times 1000$). In this case, hepatitis B is responsible from Mallory-Denk Bodies.

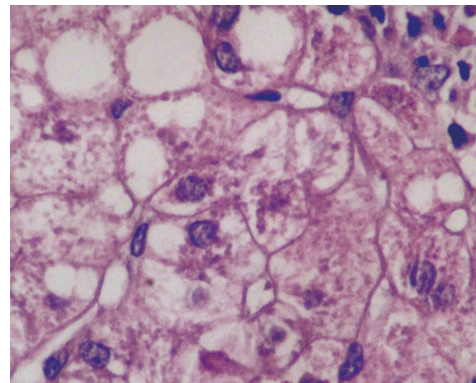


Figure 5 Tiny and uniform Mallory-Denk Bodies was seen in patients with nonalcoholic steatohepatitis ($\times 1000$).

and inflammation, Type 3: Fat and ballooned hepatocytes, and Type 4: Fat and ballooned hepatocytes and either MDB or fibrosis.

The Kleiner system is widely accepted and, as such, has superseded the previous classification system (Matteoni *et al*'s system)^[6]. Although this system includes several parameters, there are four main histologic features as steatosis, inflammatory infiltration, ballooning degeneration of hepatocytes, and fibrosis are more important than others. Also, significantly, presence or otherwise of MDB is of much less consequence in the Kleiner system.

Frequency of MDB is very variable, reported from 7% to 90% in adult patients with NAFLD^[7-18]. Furthermore, it was not demonstrated or uncommon in pediatric patients with NAFLD^[17,18].

DIAGNOSTIC CRITERIA OF MDB

Technical difficulties on the showing of MDB may be the most important reason for the differences. To better understand this issue and its contradictions, we must be aware of some basic knowledge about MDB. MDB are intracellular depositions in ballooned hepatocytes which reflects a peculiar morphological manifestation of liver cell injury^[19,20].

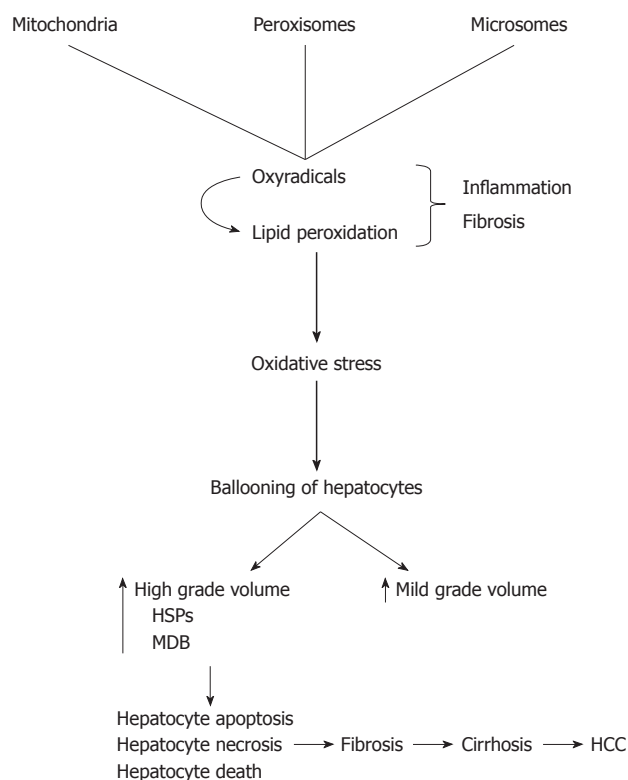


Figure 6 Shows development of Mallory-Denk Bodies in ballooned hepatocytes. MDB: Mallory-Denk Bodies; HSPs: Heat shock proteins; HCC: Hepatocellular carcinoma.

Three stages are demonstrated according to the development rank of MDB in ballooned hepatocytes: (1) misfolded proteins such as CKs, Ub, protein p62, and high and low molecular weight HSPs; (2) pre-MDB (young/tiny form) positive ballooned hepatocytes; and (3) mature or well-established MDB positive ballooned hepatocytes.

Misfolded proteins present the earliest stage of MDB and induced in response to a variety of cellular injuries. They are indicators of cellular dysfunction. Normally, these potentially harmful proteins are targeted by covalent attachment of multi-Ub chains. Then, a protective mechanism, which is the Ub-proteasome pathway, eliminates these products. When this control system fails, the CKs become accumulated in MDB along with HSPs, Ub, tissue transglutaminase, and p62. Then, mature MDB in ballooned hepatocytes is well established. There are two possible ways for this pathway to fail: (1) production of these misfolded proteins exceeding the capacity of this protective system (overexpression); and (2) inhibition of this pathway.

Pathogenesis of MDB is thought to include lipid peroxidation, oxidative stress, free radicals, bile retention, defective protein synthesis and copper accumulation (Figure 6). There are many modalities to detect MDB such as immunostaining using cytokeratin and Ub, and special staining methods for other misfolded proteins. The other one, the historically important hematoxylin and eosin, is not suitable for detecting misfolded proteins or small/tiny MDB. Almost all previous studies interested in NAFLD used hematoxylin and eosin staining for show-

ing both mature and young forms of MDB. This is a cost effective and an easily available method. Furthermore, there is more evidence and experience about its safety in the medical literature. On the other hand, the immunostaining method is relatively new and expensive. Immunostaining demonstrates not only mature form of MDB but also both its young forms and misfolded proteins such as CKs, Ub, p62 protein and HSPs in ballooned hepatocytes. The question is which one is the best to use in general practice and clinical trials? We have to look at the current medical literature to give the correct answer for this question. However, there is no growing body of evidence on this issue. A study from Denmark used immunostaining for the cell stress protein Ub in 148 fine needle liver biopsies, which included 88 biopsies from patients with clinically diagnosed or suspected alcoholic liver disease and 60 selected biopsies from non-alcoholics^[20]. They showed that MDB in ballooned hepatocytes by both hematoxylin and eosin and immunostaining (Ub) in all of 33 biopsies with alcoholic hepatitis. Of the 55 biopsies from alcoholic patients without alcoholic hepatitis (without MDB or pre-MDB in hematoxylin and eosin stained sections), Ub (+) cells were found in eight (14.5%) by immunostaining. Finally, they studied 60 selected biopsies from non-alcoholic patients, and demonstrated a few Ub (+) cells in two out of ten patients with PBC, but none in biopsies with hepatitis. According to the study results, as Ub/immunostaining is a highly sensitive and specific tool in the detection of MDB and MDB precursors in alcohol-using patients, its use in other forms of hepatitis is limited.

p62 is another misfolded protein and encoded by an immediate-early response gene that rapidly responds to a variety of extracellular signals, particularly oxidative stress. Then, protein p26 binds non-covalently to Ub.

These results may also represent a sampling error of liver biopsy. The method of liver biopsy we chose (such as percutaneous or ultrasound guided or CT guided or laparoscopic), the type of biopsy needle, the average length of the biopsies performed, and how many samples are obtained or number of biopsies performed would be important.

POSSIBILITIES IN THESE DIFFERENCES AND MANAGEMENT

Both environmental factors and genetic differences between the study groups may play a role in these differences^[21]. These remarkable differences may be induced by different levels of alcohol consumption and an incomplete patient history of alcohol consumption in these studies. Ignored alcoholic liver disease is a significant cause of MDB in some. Incomplete examination of Wilson's disease, such as just measuring serum ceruloplasmin levels or with no examination of hepatitis C virus infection, may play a role. This is especially relevant with Wilson's disease and hepatitis C because both microvesicular and macrovesicular fatty changes and glycogen nuclei are shown in both diseases.

ALCOHOLIC LIVER DISEASE

MDB is found in 70% to 75% of patients with alcoholic liver disease^[22,23]. Men and women who drink more than 80 g and 40 g of ethanol/d, respectively were accepted at substantial risk for the development of liver disease in the previous studies. Recent studies showed that the risk of liver disease begins at 30 g of ethanol/d^[24]. This finding has led to a general recommendation that the maximal safe level of ethanol consumption is 20 g/d or two “drinks” per day for men and 10 g/d for women. However, it should be kept in mind that disease severity does not correspond to classic dose dependency.

Although one of the major criteria for the diagnosis of NAFLD is no excessive alcohol consumption in all previous case series, there has been no certain agreement on this issue, and a wide range of alcohol consumption was generally allowed. Thus, these remarkable differences on the frequency of MDB might be explained with the generously allowed alcohol consumption in previous series. It must also be considered that patients sometimes underreport ethanol intake.

CONCLUSION

Frequency (MDB) is reported as very variable. Both environmental factors and genetic differences between the study groups, and technical difficulties for showing of MDB and its early stage components may play a role in these remarkable differences.

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Asymmetric dimethylarginine: A novel biomarker of gastric mucosal injury?

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Abstract

Nitric oxide (NO), a multifunctional endogenous gas molecule, is metabolized from L-arginine by enzymatic reaction in the presence of nitric oxide synthase. NO, an important gas signaling molecule, is a gastric mucosa protective factor that contributes significantly to maintain normal gastric mucosa integrity. NO increases gastric mucosa blood flow, regulates the secretion of mucus and bicarbonate, and inhibits the secretion of gastric juice. Asymmetric dimethylarginine (ADMA) has been identified as the major endogenous inhibitor of nitric oxide synthase. The function of ADMA is to decrease NO production *via* inhibiting nitric oxide synthase activity. Besides inhibiting NO synthesis, ADMA also directly induces oxidative stress and cell apoptosis, and participates in inflammation reaction. Its systemic accumulation was observed in conjunction with several cardiovascular and metabolic diseases. ADMA also mediates gastric ulcer injury induced by ethanol, stress, *helicobacter pylori* and indomethacin. The mechanism of ADMA directly producing adverse effect in gastric mucosa is incompletely understood. It is widely accepted that NO bioavailability decrease is the majority reason. Promotion of apoptosis

and aggravation of inflammation may be other important mechanisms of ADMA-induced gastric injury. ADMA might be a novel clinical and experimental biomarker related to gastric mucosa disorder. Although therapeutic tool targeting to ADMA is available in multiple cardiovascular diseases, it is unknown in gastrointestinal disease. The strategy to inhibit ADMA is beneficial to gastric ulcer induced by ethanol in rats. Thus, ADMA might be a candidate of therapeutic target in gastric mucosa damage.

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Key words: Asymmetric dimethylarginine; Mucosal injury; Nitric oxide

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INTRODUCTION

Nitric oxide (NO), a multifunctional endogenous gas molecule, is metabolized from L-arginine by enzymatic reaction in the presence of NO synthase (NOS)^[1]. It is accepted as the most critical messenger and functional executant in a variety of biologic activities. Numerous researches have reported the beneficial effects of NO, including endothelium protection, inhibition of local inflammation and cell pathoproliferation^[2]. NO bioavailability is most directly regulated by the abundance and/or activity of NOS. NOS can be competed by substrates, endogenous L-arginine and asymmetric dimethylarginine (ADMA)^[1]. The function of ADMA is to decrease NO production *via* inhibiting the NOS activity. ADMA is derived from the catabolism of

proteins containing methylated arginine residues^[3]. Arginine methylation of cellular proteins is catalyzed by protein arginine methyltransferases^[3]. ADMA is hydrolyzed by dimethylarginine dimethylaminohydrolase (DDAH)^[3].

Growing evidence has shown that excessive accumulation of ADMA can decrease NO bioavailability in cells/tissues, induce domain dysfunctions or produce detrimental effects in multiple systems^[4]. Besides inhibiting NO synthesis, ADMA can directly induce oxidative stress and cell apoptosis, and participate in the inflammation reactions^[5,6]. It is well documented that ADMA is involved in a wide range of cardiovascular disorders. ADMA impairs endothelial functions, thus leading to hypertension, atherosclerosis, coronary heart disease, diabetes mellitus, pulmonary hypertension and renal failure^[7,8]. Although it is well established that ADMA is an independent risk factor of cardiovascular diseases, the pathological role of ADMA in other diseases is still unknown, including gastric diseases.

NO AND GASTRIC MUCOSAL PROTECTION

Gastric mucosal diseases, including gastric ulcers, affect 25%-30% of the world's population. The mechanism of gastric mucosal damage and protection is yet unclear. NO, an important gas signaling molecule, is a gastric mucosal protective factor that contributes significantly to maintaining normal gastric mucosal integrity^[9]. Gastric mucosa is composed of gastric epithelial cells and glands. Gastric epithelial cells can secrete multiple active substances, including NO. There is substantial evidence that NO may be an important defendant factor of gastric mucosa^[10]. In the animal models of gastric mucosal injury induced by ethanol, endotoxin, ischemia/reperfusion, cold-stress, water immersion restraint, indomethacin and aspirin, NO exerts protective effects on gastric mucosal integrity^[11-13]. The mechanisms underlying the protective effects of NO could be explained as follows: (1) NO increases gastric mucosal blood flow; (2) NO regulates the secretion of mucus and bicarbonate, which compose the first level of gastric tissue defense; and (3) NO inhibits the secretion of gastric juice^[14,15].

ADMA AND GASTRIC MUCOSAL DAMAGE

Helicobacter pylori (*H. pylori*) infection is a definitive cause of gastroduodenal ulcer. Epidemiological analysis has shown that 70%-80% gastric ulcers and 95%-100% duodenal ulcers are attributed to *H. pylori* infection. In a clinical investigation of *H. pylori*-positive and -negative volunteers, *H. pylori* infection was found to increase the tissue contents of ADMA^[16]. More importantly, a laboratory research has shown that proteolysis of *H. pylori* extract also results in a substantial accumulation of ADMA, indicating that *H. pylori* infection must be taken into account as a cause of increased ADMA levels^[17]. Strong associations have also been found between ADMA and gastric mucosal dysfunction. Exogenous administration with ADMA inhibits the

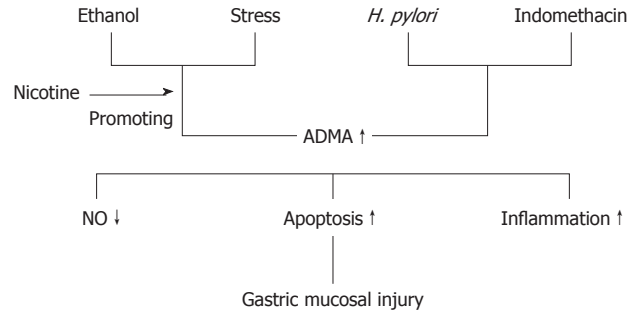


Figure 1 The hypothesized role of asymmetric dimethylarginine in gastric mucosal injury and the underlying mechanisms. ADMA: Asymmetric dimethylarginine; NO: Nitric oxide.

mucosal alkaline response to acid exposure, which is regarded as one of the important beneficial factors of gastric mucosa complaint. And an increase in ADMA mediates the water extract of *H. pylori*-induced acid-base imbalance in duodenal tissues^[17]. In our *in vitro* experiment, we found that intervention of *H. pylori* stimulated ADMA release from cultured gastric epithelial cells^[18]. We also observed that *H. pylori* infection markedly exacerbated ADMA-produced gastric tissue injury in rats^[19].

Besides *H. pylori* invasion, gastric mucosal function is also disturbed by other factors, such as ethanol, indomethacin and cold-stress. Our recent studies have explored the role of ADMA in gastric mucosal injury induced by other causes in addition to *H. pylori* infection. The results have shown that ADMA levels are increased in gastric juice in three separate gastric mucosal models, including ethanol, indomethacin and cold-stress initiated mucosal injury, accompanied with a decrease in NO level and DDAH activity^[19]. Pretreatment with L-arginine, an antagonist of ADMA, markedly reduces the degree of gastric injury and elevates NO bioavailability. Administration of exogenous ADMA significantly exacerbates gastric injury, concomitantly with a decrease in plasma level of NO. These findings indicate that ADMA may participate in the gastric mucosal injury process induced by various deleterious factors. Multiple exogenous NOS inhibitors, such as L-NMA, L-NAME and L-NMMA intensify gastric mucosal injury by decreasing gastric mucosal blood flow, while NO donor (nitroprusside sodium) or substrate (L-arginine) reverses such effects^[13,20].

Cigarette smoking is one of the risk factors provoking gastroduodenal ulceration^[21]. It increases both the incidence and relapse rate of peptic ulcer diseases and delays ulcer healing. Besides reducing bioavailability of NO, nicotine, a major component of tobacco, aggravates gastric mucosal injury due to some factors such as ethanol or stress^[22]. Recently, we have found that in the rats treated with ethanol, the ulcer index and ADMA level were increased and the NO level was decreased, and these effects of ethanol were augmented by pre-treatment with nicotine^[23]. Nicotine alone did not show significant impact on ulcer index, ADMA level and NO level. *In vitro* incubation of epithelial cells with ethanol induced cell injury and increased ADMA level in the cultural medium, an effect that was amplified in the presence of nicotine. Therefore, the

accelerated effect of nicotine on gastric mucosal dysfunction is associated with endogenous ADMA.

The mechanism of ADMA directly producing adverse effect in gastric mucosa is incompletely understood. It is widely accepted that NO bioavailability decrease is the major reason. Apoptosis reaction produced by ADMA has been observed in different types of cells, such as endothelial cell and smooth muscle cell^[6,24]. Cell apoptosis has also been observed in cultured gastric epithelial cells when exposed to an exorbitant concentration of ADMA^[23]. ADMA could directly induce the production of inflammatory cytokines tumor necrosis factor (TNF)- α and soluble intercellular adhesion molecule-1 *via* activation of p38 MAPK and ERK1/2 pathways in cultured endothelial cells^[25]. We deduce that inflammatory reaction may be another important factor for ADMA-induced gastric injury because TNF- α level, the marker of inflammation, is significantly elevated when cells are damaged by ADMA in gastric mucosal epithelial cells. The hypothesized role of ADMA in gastric mucosal injury and the underlying mechanisms are summarized in Figure 1.

PERSPECTIVES AND CLINICAL IMPLICATIONS

ADMA might be a novel clinical and experimental biomarker related to gastric mucosal disorder. The mechanisms of impairment involve NO generation inhibition, inflammatory reaction and apoptosis promotion. Although the therapeutic tool targeting ADMA is available in multiple cardiovascular diseases, it is unknown in gastrointestinal diseases. The strategy to inhibit ADMA is beneficial to the treatment of gastric ulcer induced by ethanol in rats^[26]. Thus, ADMA might be a candidate of therapeutic target in gastric mucosal damage.

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Soluble ST2: A new and promising activity marker in ulcerative colitis

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with the severity of ulcerative colitis (UC) and serum levels of pro-inflammatory cytokines, and to demonstrate the predictive power of sST2 levels for differentiation between active and inactive UC.

METHODS: We recruited 153 patients: 82 with UC, 26 with Crohn's disease (CD) and 43 disease controls [non-inflammatory bowel disease (IBD)]. Subjects were excluded if they had diagnosis of asthma, autoimmune diseases or hypertension. The serum levels of sST2 and pro-inflammatory cytokines [pg/mL; median (25th-75th)] as well as clinical features, endoscopic and histological features, were subjected to analyses. The sST2 performance for discrimination between active and inactive UC, non-IBD and healthy controls (HC) was determined with regard to sensitivity and specificity, and Spearman's rank correlation coefficient (r). To validate the method, the area under the curve (AUC) of receiver-operator characteristic (ROC) was determined (AUC, 95% CI) and the total ST2 content of the colonic mucosa in UC patients was correlated with circulating levels of sST2.

RESULTS: The serum sST2 value was significantly higher in patients with active [235.80 (90.65-367.90) pg/mL] rather than inactive UC [33.19 (20.04-65.32) pg/mL], based on clinical, endoscopic and histopathological characteristics, as well as compared with non-IBD and HC ($P < 0.001$). The median level of sST2 in CD patients was 54.17 (35.02-122.0) pg/mL, significantly higher than that of the HC group only ($P < 0.01$). The cutoff was set at 74.87 pg/mL to compare active with inactive UC in a multicenter cohort of patients. Values of sensitivity, specificity, and ability to correctly classify UC, according to activity, were 83.33%, 83.33% and 83.33%, respectively. The AUC of the ROC curve to assess the ability of this molecule to discriminate between active vs inactive UC was 0.92 (0.86-0.97, $P < 0.0001$). The serum levels of sST2 in patients with UC significantly correlated with endoscopic and histo-

Abstract

AIM: To correlate circulating soluble ST2 (sST2) levels

pathological scores ($r = 0.76$ and $r = 0.67$, $P < 0.0001$, respectively), and with the pro-inflammatory cytokine, tumor necrosis factor- α ($r = 0.69$ and $r = 0.61$, respectively, $P < 0.0001$). Interestingly, we found a direct correlation between total intestinal ST2 content and serum levels of sST2, adjusted to endoscopic activity score in patients with mild ($r = 0.44$, $P = 0.004$), moderate ($r = 0.59$, $P = 0.002$) and severe disease ($r = 0.82$, $P = 0.002$). Only patients with inactive UC showed no significant correlation ($r = 0.45$, $P = 0.267$).

CONCLUSION: sST2 levels correlated with disease severity and inflammatory cytokines, are able to differentiate active from inactive UC and might have a role as a biomarker.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Soluble ST2; Biomarkers

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INTRODUCTION

Inflammatory bowel diseases (IBDs) belong to the group of chronic diseases that cause intestinal inflammation. Ulcerative colitis (UC) and Crohn's disease (CD) are the two most important diseases in this group. Their characteristics are mainly episodes of active inflammation or remission. In order to provide a differential diagnosis of these diseases, it is necessary to know the clinical, endoscopic, histological, radiologic and serologic characteristics, as well as their course throughout time^[1].

Currently, classifications of IBD are based on epidemiologic (age, gender, race), clinical (activity rate, localization and phenotype) and genetic [single nucleotide polymorphism (SNP)]^[2,3] parameters, and the presence of biological markers^[4,5]. However, due to the high percentage of non-classifiable IBD (10%-15%) and the difficulty of a differential diagnosis, it has become necessary to search for new markers for these diseases.

One ideal characteristic of an IBD biomarker is the specificity; however, it also has to be easy to detect, tests must be minimally invasive, low-cost, quick to perform and replicable across laboratories^[6,7]. In addition, the

biomarker should be able to identify individuals at risk of developing the disease, detect the activity, monitor the effect of the treatment and, finally, have a prognostic value for the reactivation of the disease^[7,8]. Current biomarkers for IBD include serological levels of specific antibodies (ASCA, ANCA, anti-OmpC, anti-Cbir, anti-glycans)^[9-12], serum (CRP and cytokines)^[13-15] and fecal proteins (calprotectin and lactoferrin)^[5,7,16-20]. Nevertheless, the majority of these markers show a low sensitivity and/or specificity, and they cannot reflect the real intestinal damage.

In this context, sST2 protein has recently been identified as a new and reliable biomarker of heart failure^[21-26]. High serum levels of sST2 have been described in patients with chronic inflammatory diseases, such as autoimmune diseases^[27,28] and asthma^[29].

ST2 belongs to the interleukin (IL)-1R super-family, is coded in human chromosome 2 and is expressed as two splice variants: one membrane bound, ST2L, which is a receptor of IL-33; and a soluble protein, sST2^[30-32].

Recently, in our laboratory, we have described for the first time increased levels of sST2 in serum and total ST2 in the colonic mucosa in IBD patients, and also its distribution in epithelial and infiltrating cells from colonic mucosa. In addition, we showed that serum sST2 levels significantly correlate with total ST2 levels in colonic mucosa^[33]. Supporting our results, other groups also have shown evidence that the ST2/IL-33 system could be participating in the development of IBD^[34-36].

To date, there are no studies that correlate levels of sST2 with severity of the UC.

The aims of the present study were to determine in another cohort of UC patients whether serum sST2 and intestinal total ST2 levels correlate with the severity of the disease, based on endoscopic and histological activity rates, and with serum levels of pro-inflammatory cytokines.

MATERIALS AND METHODS

Participants were recruited from the Gastroenterology Departments at "Clínica Las Condes", "Hospital Clínico de la Universidad de Chile" and "Hospital Clínico de la Pontificia Universidad Católica de Chile", respectively. Patients were diagnosed based on standard clinical, endoscopic and histological criteria. The study was approved by the Ethics Committee/Ethics Review Board of each participating center, and all patients signed an informed consent prior to their participation in this study.

During the study process, between January 2008 and December 2009, 153 patients were subjected to colonoscopy. Procedures were carried out by gastroenterologists with more than 5 years of experience in colonoscopy (co-authors RQ, MA-L), and findings were classified according to the clinical criteria of the Montreal Classification. Inclusion criteria for the study were: IBD diagnosed patients, > 18 years, blood specimens collected just before colonoscopy, biopsies taken and informed consent.

Exclusion criteria were: non-classifiable inflammatory disease, indeterminate colitis, infectious ileocolitis, asthma, history of autoimmune diseases, celiac disease and hypertension.

Patients were grouped based on endoscopic and histological criteria: Group UC ($n = 84$) and CD ($n = 26$), and non-IBD controls (irritable bowel syndrome, colorectal cancer, family history of colorectal cancer, diverticular disease and chronic diarrhea; $n = 43$). In addition, a group of healthy subjects ($n = 40$, between 18 and 45 years old) were included to determine reference levels of sST2.

A 5 mL blood specimen was obtained from each patient, and 3 to 4 biopsies were immediately frozen in liquid nitrogen and stored at -80°C until analysis. From the healthy subjects, only a blood sample was obtained for analysis.

In the case of UC, endoscopic activity was determined in the most swollen area using the endoscopic Mayo Score^[37]. In the case of CD, clinical activity was determined according to the Harvey-Bradshaw Index (HBI)^[38], and for endoscopic activity, we used the Simple Endoscopic Score for Crohn's Disease (SES-CD)^[39]. Histopathological score was used for the evaluation of intestinal inflammation in both diseases. Each biopsy was graded on a scale of 0-3 (0 = normal; 1 = mild; 2 = moderate; 3 = severe and included those patients with active ulceration) according to Gomes *et al.*^[40].

Quantification of serum sST2 and total intestinal ST2 levels

Levels of sST2 and total intestinal ST2, in serum and protein extract of colonic mucosa, respectively, were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit for human ST2 (DuoSet, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Serum samples obtained from 5 mL of blood were subjected to a treatment with protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein extracts were obtained from each sample by homogenization using a lysis buffer supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Basel, Switzerland) and subsequent disruption by sonication. Levels of total intestinal ST2 were adjusted to the total protein concentration determined by Bradford protein assay. All samples were analyzed in duplicate and each determination was expressed in pg/mL. The detection limit of the technique, provided by the kit's manufacturer, is 20 pg/mL.

Measurement of serum inflammatory cytokines

Serum levels of IL-33 (Apotech, Geneva, Switzerland), IL-6 (Human IL-6 ELISA Ready-Set-Go, eBioscience, San Diego, CA, USA), and tumor necrosis factor (TNF)- α (Human TNF- α ELISA Ready-Set-Go, eBioscience) were measured using an ELISA kit, according to the manufacturer's instructions. The detection limits provided by the kit manufacturers are 5 pg/mL, 2 pg/mL and 4 pg/mL

for IL-33, IL-6 and TNF- α , respectively. Samples were prepared as previously described for ST2 determination.

Statistical analysis

Data were analyzed using the statistical software Graph-Pad Prism4 (La Jolla, CA) and are presented as average $\pm$ SD for variables with a normal distribution, and as a median (25th-75th) in the case of non-parametric distributions. Differences and significances among analyzed groups were established by multiple comparisons using the non-parametric Kruskal-Wallis tests. Further comparisons of individual groups *vs* control were performed by using Bonferroni-Dunn statistics and a 5% significance level. Sensitivity and specificity for levels of sST2, and their respective confidence intervals of 95%, were calculated according to endoscopic activity and compared to a non-inflammatory condition. The best cut-off value for sST2 that discriminated between inactive UC and active UC patients and the different levels of endoscopic activity was determined by area under the curve (AUC). Uni and bivariate analyses were carried out to determine the risk factors associated with each one of the following demographic and clinical parameters: gender, age, extent of the disease and medication at endoscopy. The associations between serum levels of sST2 and total intestinal ST2, and serum cytokines, were analyzed using Spearman's rank correlation coefficient (r). For each statistical test that was used, values of $P \leq 0.05$ were considered significant.

RESULTS

Main characteristics of IBD patients

During the study period, a total of 153 patients were recruited. Of these, 84 (54.9%) corresponded to UC, 26 (16.9%) to CD and the other 43 (28.1%) to non-IBD controls. Table 1 summarizes the main characteristics of IBD patients following the Montreal classification and also indicates gender and age distribution among groups, as well as medication at endoscopy. At the time of the procedure, 40 UC (47.6%) and 12 CD patients (46.1%) were active according to endoscopic Mayo and SES-CD criteria, respectively.

Determination of reference levels and cut-off value for sST2 in patients with IBD

The reference level of sST2 in serum, determined in the healthy subject group (HC), was 32.40 (19.00-49.00) pg/mL; in the case of the non-IBD, CD and UC groups, levels were 46.33 (26.00-74.66) pg/mL, 54.17 (35.02-122.0) pg/mL and 67.59 (30.78-199.1) pg/mL, respectively, with significant differences ($P < 0.001$) between UC *vs* HC and CD *vs* HC (Figure 1A). Due to the low number of patients with each of the CD phenotypes, such as inflammatory, penetrating and stenosing, we decided to focus on the analysis of sST2 levels restricted to the group of UC patients. Analysis of the levels of sST2 in the serum of the UC group, according to the Mayo Score of endoscopic activity (active ≥ 2) resulted in concentrations of

Table 1 Clinical characteristics of the patient groups *n* (%)

	IBD				Controls	
	UC		CD		Non-IBD	HC
No. of patients	84		26		43	40
Female (%)	48 (57.1)		13 (50)		23 (53.4)	18 (45)
Age (mean $\pm$ SD, yr)	38 $\pm$ 12.6		42.6 $\pm$ 15.5		49.8 $\pm$ 18.3	30.8 $\pm$ 5.4
Location of disease						
Ulcerative proctitis, E1	16 (19)					
Left-sided colitis, E2	16 (19)					
Extensive colitis, E3	52 (62)					
Small bowel, L1			4 (15.5)			
Colon, L2			15 (57.6)			
Ileocolonic, L3			7 (26.9)			
Disease behavior						
Inflammatory			25 (96.1)			
Stenosing			1 (3.9)			
Penetrating			0			
Index disease	0	1	2	3	≤ 8	≥ 9
Endoscopic Mayo score	8 (9.5)	40 (47.6)	25 (29.7)	11 (13.2)		
Histopathological score	8 (9.5)	35 (41.6)	32 (38.1)	9 (10.7)		
Harvey-Bradshaw Index					22 (84.6)	4 (15.4)
SES-CD					24 (92.3)	2 (7.7)
Medication at endoscopy						
No medication	14 (16.6)		5 (19.2)			
Topical 5-ASA	16 (19)		2 (7.7)			
Systemic 5-ASA	24 (28.5)		7 (26.9)			
Systemic steroids	10 (11.9)		3 (11.6)			
5-ASA + steroids	13 (15.5)		0			
5-ASA + azathioprine	7 (8.5)		4 (15.4)			
Azathioprine	0		5 (19.2)			

Data regarding location of disease, disease behavior, index disease and medication at endoscopy are represented as number of patients (%). UC: Ulcerative colitis; CD: Crohn's disease; HC: Healthy subjects; SES-CD: Simplified endoscopic activity score for Crohn's disease; 5-ASA: 5-aminosalicylic acid derivatives; IBD: Inflammatory bowel diseases.

Table 2 Sensitivity, specificity and cut-off values for sST2 in ulcerative colitis patients compared to other groups

	Inactive UC				Active UC			
	Cut-off value (pg/mL)	Sensitivity (%)	Specificity (%)	AUC (95% CI)	Cut-off value (pg/mL)	Sensitivity (%)	Specificity (%)	AUC (95% CI)
HC	33.08	54.29	52.08	0.58 (0.46-0.71)	64.25	94.29	91.67	0.98 (0.95-1.00) ^b
Non IBD	42.67	60.98	64.58	0.58 (0.45-0.70)	76.29	80.49	83.33	0.90 (0.84-0.97) ^b
iUC	-	-	-	-	74.87	83.33	83.33	0.92 (0.86-0.97) ^b

^b*P* < 0.0001. iUC: Inactive ulcerative colitis; HC: Healthy subjects; IBD: Inflammatory bowel disease; AUC: Area under the curve.

33.19 (20.04-65.32) pg/mL for inactive UC and 235.8 (90.65-367.9) pg/mL for active UC, with significant differences between active UC *vs* HC, non-IBD and inactive UC (*P* < 0.0001) (Figure 1B).

Figure 1C shows the ROC curve for sST2 levels in the active UC group in relation to the inactive UC group of patients. The optimal cut-off value estimated for sST2 that allows for the discrimination of active UC compared to inactive UC was 74.87 pg/mL. The AUC of the ROC values, cut-off, sensitivity and specificity are summarized in Table 2.

Levels of sST2, IL-33, TNF- α and IL-6 in serum and their correlation with disease activity

The range of serum sST2 concentrations for the different UC sub-groups, according to endoscopic Mayo score,

is shown in Figure 2 and Table 3. Significant differences were observed among groups with moderate (Score 2) and severe activity (Score 3) comparing to inactive (Score 0) and mild activity (Score 1) sub-groups (*P* < 0.001) (Figure 2A). Regarding the histopathological compromise of the mucosa, the serum sST2 levels were significantly higher in the severe (Score 3) and moderate (Score 2) inflammation groups compared to both normal (Score 0) and mild (Score 1) sub-groups (*P* < 0.001 and *P* < 0.01, respectively) (Figure 2B). Endoscopic and histopathological scores directly correlated with serum sST2, with *r* = 0.76 and *r* = 0.67, respectively (Table 3).

Levels of serum TNF- α in the different UC sub-groups were directly proportional to endoscopic and histopathological scores. When comparing serum sST2 and cytokine levels, only TNF- α significantly correlated, both

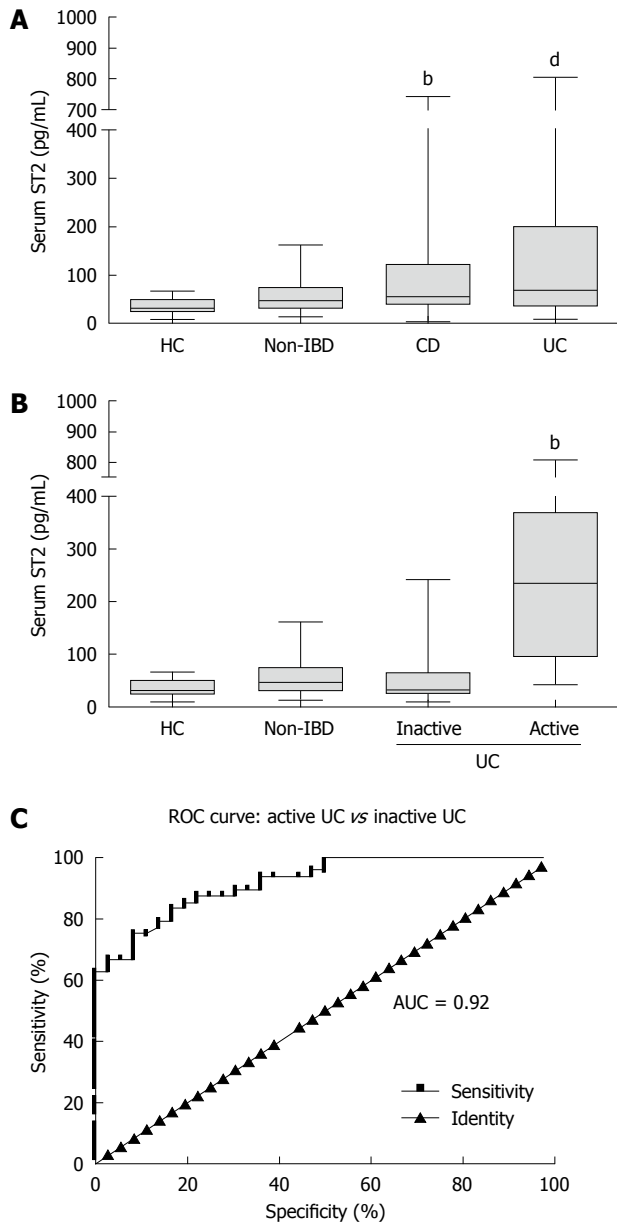


Figure 1 Distribution of serum levels of sST2 in the groups of studied patients. Box-plot showing the distribution of serum sST2 levels in healthy patients (HC) compared to non-inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) patients (A) and comparison of HC with non-IBD and inactive and active UC patients (B). Data are represented as median and percentiles (25th-75th) and significant differences in serum sST2 levels are shown between UC and CD patients vs HC (A) and between active UC and other groups (B). Receiver operating characteristics (ROC) curves for sST2 cut-off point determination (C). ROC curves illustrate the specificity and sensitivity in serum sST2 level determination to differentiate active from inactive UC. A: ^b $P < 0.01$, CD vs HC; ^d $P < 0.001$, UC vs HC; B: ^b $P < 0.001$, active UC vs HC, non-IBD and inactive UC.

with endoscopic ($r = 0.69$, $P < 0.0001$) and histopathological scores ($r = 0.61$, $P < 0.0001$) (Table 3).

Uni and bivariate analyses of serum levels of sST2, with reference to demographic and clinical parameters such as age, gender, localization of the disease and medication at the endoscopy for each one of the analyzed groups, are shown in Table 4. In addition to the activity score, significant differences were observed for localiza-

Table 3 Correlation data of endoscopic and histopathological scores with serum sST2, interleukin-33, interleukin-6, tumor necrosis factor- α levels and total intestinal ST2

Disease activity index	Endoscopic score	Histopathological score
sST2 (pg/mL)		
r	0.7624	0.6762
P -value	< 0.0001	< 0.0001
IL-33 (pg/mL)		
r	0.2869	0.1687
P -value	0.0146	0.1657
IL-6 (pg/mL)		
r	0.2315	0.0888
P -value	0.0676	0.2855
TNF- α (pg/mL)		
r	0.6961	0.6112
P -value	< 0.0001	< 0.0001
Intestinal ST2 (pg/mL per mg protein)		
r	0.6267	0.6034
P -value	< 0.0001	< 0.0001

IL: Interleukin; TNF: Tumor necrosis factor; r : Spearman's rank correlation coefficient.

tion ($P = 0.0061$) and medication ($P = 0.0067$) of the UC group (Table 4). These results show the same trend observed in Figure 2A and B, based on endoscopic and histopathological scores, regarding gender ($P < 0.0001$), localization ($P < 0.0001$) and medication with 5-aminosalicylic acid (5-ASA) ($P = 0.0005$) (data not shown). These findings demonstrate that sST2 values exclusively depend on the severity of the disease.

Levels of total intestinal ST2 correlate with disease activity scores and serum levels of sST2 in UC patients

In order to determine if the findings observed at the systemic level reflect the local damage, total intestinal ST2 was measured in colonic mucosa. Similarly to the serum sST2 levels, total intestinal ST2 levels in UC are closely distributed to activity endoscopic ($P < 0.0001$) (Figure 2C) and histopathological score ($P < 0.0001$) (Figure 2D). Significant differences were observed between moderate and severe activity sub-groups compared to inactive and mild (Figure 2C and D). Similarly, total intestinal ST2 levels significantly correlated with endoscopic ($r = 0.62$, $P < 0.0001$) and histopathological scores ($r = 0.60$, $P < 0.0001$) (Table 3), as seen for serum sST2 levels (Figure 2A and B).

Furthermore, serum sST2 levels and total intestinal ST2 directly correlate, according to endoscopic Mayo activity score, in the severe ($r = 0.82$, $P = 0.0027$), moderate ($r = 0.59$, $P = 0.0020$) and mild ($r = 0.44$, $P = 0.0045$) sub-groups (Figure 3).

DISCUSSION

Our group first reported that the ST2/IL-33 system, described in other inflammatory diseases, could be involved in the pathogenesis of IBD, because levels of ST2 and IL-33 in IBD patients were higher than in healthy sub-

Table 4 Baseline serum sST2 levels according to clinical characteristics of patients

	Serum sST2 (pg/mL), median (Percentiles 25th-75th)		
	UC	Non-IBD	HC
Gender			
Female	55.5 (29.3-150.1)	43.9 (22.1-73.1)	29.4 (17.0-40.3)
Male	99.7 (34.0-216.4)	59.7 (35.6-74.9)	36.2 (27.0-53.2)
Age (yr)			
18-24	190.8 (52.6-489.6)	63.2 (31.7-74.9)	29.0 (17.0-45.0)
25-34	66.2 (30.4-313.3)	46.3 (18.5-116.6)	32.4 (16.9-40.8)
35-44	125.4 (37.0-182.9)	48.1 (32.2-67.8)	44.3 (27.9-59.6)
45-54	48.2 (24.9-116.4)	43.6 (26.0-72.4)	51.0 (35.7-65.0)
≥ 55	51.3 (19.9-90.5)	53.0 (25.9-78.8)	0
Location of disease			
Ulcerative proctitis, E1	56.5 (22.4-141.6)		
Left-sided colitis, E2	35.8 (19.4-66.2)		
Extensive colitis, E3	110.2 (34.0-345.8) ^a		
Medication at endoscopy			
No medication	36.4 (19.6-105.6)		
Topical 5-ASA	39.7 (20.4-75.7)		
Systemic 5-ASA	59.0 (23.6-141.7)		
Systemic steroids	242.4 (124.9-349.1) ^a		
5-ASA + steroids	125.4 (50.0-417.9)		
5-ASA + azathioprine	41.1 (68.9-109.0)		
Azathioprine	0		

^a $P \leq 0.05$ vs other groups in the analysis. UC: Ulcerative colitis; HC: Healthy subjects; IBD: Inflammatory bowel disease; 5-ASA: 5-aminosalicylic acid derivatives.

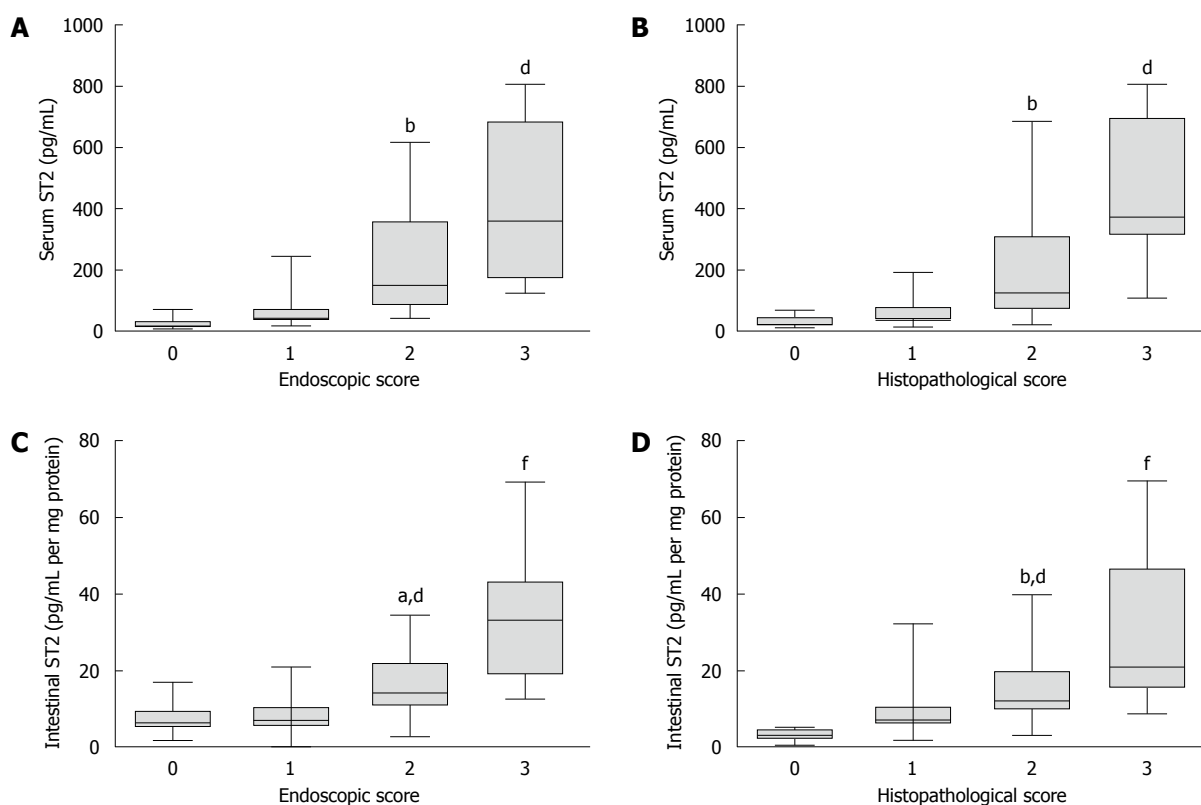


Figure 2 Analysis of serum sST2 and total intestinal ST2 levels in ulcerative colitis patients according to endoscopic and histopathological activity. Distribution of serum sST2 and total intestinal ST2 levels in ulcerative colitis (UC) patients according to the 4 rank Endoscopic Mayo Activity Score (A and C) (Activity: 0 = inactive; 1 = mild; 2 = moderate; 3 = severe) and histopathological score (B and D) (Degree of inflammation: 0 = normal; 1 = mild; 2 = moderate; 3 = severe with active ulceration). Data are represented as median and percentiles (25th-75th). Serum sST2 levels are significantly different among UC patient sub-groups of moderate and severe activity in relation to inactive and mild activity, independent of the score used. Total intestinal ST2 levels directly correlate with endoscopic activity (C) and degree of mucosal inflammation (D). A: ^b $P < 0.001$, 2 vs 0 and 1; ^d $P < 0.001$, 3 vs 0 and 1; B: ^b $P < 0.01$, 2 vs 0 and 1; ^d $P < 0.001$, 3 vs 0 and 1; C: ^a $P < 0.05$, 2 vs 1; ^d $P < 0.01$, 2 vs 0; ^f $P < 0.001$, 3 vs 0 and 1; D: ^b $P < 0.01$, 2 vs 1; ^d $P < 0.001$, 2 vs 0; ^f $P < 0.001$, 3 vs 0 and 1.

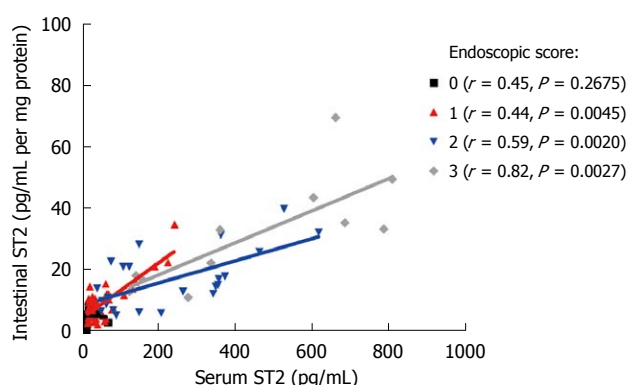


Figure 3 Graphic representation of the direct correlation between serum sST2 and total intestinal ST2 levels according to endoscopic activity scores. Activity: 0 = inactive; 1 = mild; 2 = moderate; 3 = severe. The trend lines for each analyzed group are shown. *r*: Spearman's rank correlation coefficient.

jects^[33]. Recently, Pastorelli *et al*^[34] also reported an increase in ST2/IL-33 system components in patients with IBD, both in the colonic mucosa as well as in serum. However, they reported that the circulating IL-33 levels in IBD patients were higher than our results and than in other diseases, even higher than those shown in sepsis^[34]. Another two articles in the field^[35,36] also confirmed elevated IL-33 expression in the mucosa of active IBD patients, with the limitation that these observations were conducted mainly at mRNA level, but ST2 levels were not studied.

The present study demonstrates, for the first time, a direct association between serum levels of sST2 protein and the degree of endoscopic and histopathological activity in UC.

Currently, a large number of serological, fecal or miscellaneous molecules have been proposed as indirect markers of IBD severity: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and even antineutrophil cytoplasmic antibodies (ANCA) and anti-*Saccharomyces cerevisiae* antibodies (ASCA). However, lately they have become less useful for the diagnosis and prognosis of the diseases. This is mainly due to a low sensitivity and specificity to intestinal inflammation^[41] and these markers do not allow accurate differentiation between IBD and other intestinal diseases, nor do they discriminate activity status. Some molecules detected in serum are rather systemic markers that in general do not show inflammatory bowel processes^[12,42]. The use of a serum inflammation marker that reflects the intestinal damage would be helpful for the management and prognosis of IBD patients. Fecal molecules, such as calprotectin and lactoferrin, represent an inflammatory neutrophilic process of the intestinal mucosa^[16]. However, these are also non-specific markers of inflammation, which are increased in organic intestinal diseases such as diverticular disease^[43,44] polyposis^[45] and colorectal cancer^[46,47].

Serum levels of sST2 allow for a highly valuable discrimination between UC patients and healthy subjects; however, this efficacy is reduced when trying to differentiate UC from organic intestinal diseases presenting any

degree of inflammation. Alternatively, sST2 would allow, as happens with fecal calprotectin, the differentiation between UC and functional diseases, such as irritable bowel syndrome, chronic diarrhea and abdominal pain^[5,19,48]. Many studies have shown that calprotectin significantly correlates with endoscopic and histological activity scores in CD and UC patients^[49-51]. Calprotectin level decreases during clinical remission, which could be related to endoscopic mucosal healing^[42,49,52], and consequently is considered a predictor of IBD reactivation. Serum sST2 levels allow for the differentiation between active and inactive UC with a high sensitivity and specificity. The cut-off value determined (74.87 pg/mL) permits the differentiation between active and inactive UC patients, as well as healthy subjects.

Similarly to fecal calprotectin, serum sST2 levels from UC patients significantly correlated with endoscopic ($r = 0.76$), as well as histopathological score ($r = 0.67$). Serum IL-33 level, another of the cytokines evaluated, did not show a direct relationship with disease activity; this might be due to the low levels detected compared to sST2, despite being the specific ligand of ST2. Serum sST2 levels in UC patients correlate with activity scores comparable with TNF- α , a commonly used serum inflammation marker. These characteristics result in the proposition of sST2 as an appropriate marker of inflammatory activity degree in UC. However, correlation of serum sST2 levels has to be achieved with other activity biomarkers previously associated with IBD, such as CRP or calprotectin.

In the case of CD patients, the analysis of serum sST2 values showed similar tendencies to those in UC, in relation to control patients (Figure 1A). The low incidence of CD in Chile^[53], in addition to the exclusion criteria used in our study, account for the low number of CD patients included. Future studies will allow us to determine the association of sST2 with the inflammatory, stenosing and penetrating phenotypes of CD so as to support the concept that sST2 may also be applicable as a biomarker in CD.

Recently, ST2 has been described as a biomarker for heart failure, as serum levels correlate with hemodynamic variables, cardiac damage (BNP and pro-BNP) and inflammatory markers (CRP)^[22,23,54,55]. In those studies, serum sST2 levels increase after myocardial infarction^[21,56]; hence patients with a history of cardiopathies and hypertension were excluded.

In addition, some biochemical properties of sST2 support its characteristic as a reliable biomarker in UC, mainly based on its stability^[57] and limited dependence on epidemiological and clinical factors, such as age, gender and diet^[58].

In our study, serum sST2 levels in healthy subjects were similar to those described previously [32.4 (19-49) pg/mL vs 49 (4-89) pg/mL]^[54]. In addition, serum sST2 levels were higher in males than in females, and slightly increased between 18 and 24 years in age, as previously described^[58]. However, when considering serum sST2 levels together with endoscopic activity, adjusted by gender, the distribution remained the same; therefore, we conclude that sST2

levels do not depend on these factors.

Therapeutic strategies for IBD patients are determined according to severity and localization of the affected area. UC patients with pancolitis presented higher serum sST2 levels in relation to proctitis or left-sided colitis. UC patients receiving systemic corticoids showed an increased sST2 level when compared to other treatments. Due to the low number of patients, we were not able to determine whether corticoids affect the sST2 concentration and its correlation with activity scores. However, in UC patients, ST2 levels did not show an association with mesalazine (5-ASA) treatment and in those patients sST2 levels follow activity degree of the disease. One of the most important qualities of a biomarker is that it has to be used in clinical practice and not be affected by drug therapy^[12]. One of the main limitations of calprotectin as an IBD marker is the influence of non-steroidal anti-inflammatory drugs on its level, as previously shown^[59-61]. In our study, 66.3% of IBD patients were receiving 5-ASA treatment, so measurement of calprotectin in those patients may be inconclusive.

Total ST2 levels in the colonic mucosa of UC patients significantly correlated with endoscopic and histopathological activity scores. In addition to the fact that total intestinal ST2 levels are directly associated with serum sST2 levels, these findings verify it as a new and promising UC activity biomarker. The relation between serum sST2 and inflammatory bowel activity would allow, in the future, the avoidance of a colonoscopic procedure in patients that do not require it. Association studies between ST2 and other biomarkers, such as calprotectin, may confirm its use.

It is possible that sST2 not only acts as a marker of UC activity; functions attributed to sST2 account for a role as an immunomodulator in inflammatory processes. At the cellular level, sST2 has been described as an inhibitor of IL-33/ST2L signaling^[62], which causes polarization of naive T cells into Th2, and further, the production of IL-5 and IL-13 that are associated with UC^[63,64]. On the other hand, ST2L activation with IL-33 stimulates TNF- α , IL-6 and IL-8 secretion in mast cells^[65,66] and, together with IgE, stimulates degranulation^[67]. The increase of sST2 during periods of inflammation may be involved in the control of the immune response associated with IBDs such as UC.

In summary, we demonstrated that serum sST2 levels allow for the effective differentiation between the endoscopic activity degrees of UC. Determining whether serum sST2 levels could have any prognostic value for UC (and possibly for CD), whether sST2 levels could monitor the treatment impact on endoscopic mucosal healing, and whether they could predict the risk of complications in IBD course or need of surgery, are some of the questions that should be answered by further studies.

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COMMENTS

Background

Inflammatory bowel diseases (IBDs) belong to the group of chronic diseases that cause intestinal inflammation. Ulcerative colitis (UC) and Crohn's disease are the two most important diseases in this group. Their characteristics are mainly episodes of active inflammation or remission. Currently, classifications of UC are based on epidemiologic, clinical and genetic parameters, and the presence of biological markers. Therapeutic strategies for UC patients are determined according to severity and localization of the affected area.

Research frontiers

To date, there are no studies that correlate levels of soluble ST2 (sST2) with severity of UC. It is possible that sST2 not only acts as a marker of UC activity; functions attributed to sST2 account for a role as immunomodulator in inflammatory processes. At the cellular level, sST2 has been described for the first time increased levels of sST2 in the serum and total ST2 in the colonic mucosa of UC patients. The increase of sST2 during periods of inflammation may be involved in the control of the immune response associated with IBD.

Innovations and breakthroughs

Soluble ST2 protein has been identified as a new and reliable biomarker of heart failure. High serum levels of sST2 have been described in patients with chronic inflammatory diseases, such as autoimmune diseases and asthma. Recently, in their laboratory, the authors have described for the first time increased levels of sST2 in the serum and total ST2 in the colonic mucosa of UC patients. In this study, we show that serum sST2 levels significantly correlate with total ST2 levels in the colonic mucosa. Supporting our results, other groups also have shown evidence that the ST2/IL-33 system participates in the development of IBD.

Applications

The relation between serum sST2 and inflammatory bowel activity would allow, in the future, avoidance of colonoscopy procedures in patients that do not require them. In addition, some biochemical properties of sST2, such as its stability, support its characteristic as a reliable biomarker in UC. If sST2 levels decreased during clinical remission, these could be related to endoscopic mucosal healing, and therefore be considered a predictor of UC reactivation.

Terminology

ST2 belongs to the IL-1R super-family, is coded in human chromosome 2 and is expressed as two splice variants: one membrane bound, ST2L, which is a receptor of IL-33; and a soluble protein, sST2.

Peer review

The authors examined the expression of components of the ST2/IL-33 system in serum and colonic mucosa in UC patients and correlated levels of sST2 with severity of the disease. The study revealed that sST2 levels are able to differentiate active from inactive UC and correlate with endoscopic and histopathological activity scores. In addition to the fact that total intestinal ST2 levels are directly associated with serum sST2 levels, these findings verify that circulating sST2 levels may play an important role as a new and promising biological marker in UC.

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Parallel decline of CD8+CD38+ lymphocytes and viremia in treated hepatitis B patients

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Abstract

AIM: To assess the peripheral T lymphocyte subsets in chronic hepatitis B virus (HBV) infection, and their dynamics in response to adefovir dipivoxil monotherapy.

METHODS: Proportions and absolute counts of peripheral natural killer cells, B cells, CD8+, CD4+, CD8+CD38+, CD8+CD28+ and CD4+CD28+ T cells were determined using three-color flow cytometry in chronic hepatitis B patients ($n = 35$), HBV carriers ($n = 25$) and healthy controls ($n = 35$). Adefovir dipivoxil was initiated in 17 chronic hepatitis B patients who were regularly followed for 72 wk, during which period the T cell subsets and serum viral load were measured at each follow-up point.

RESULTS: The peripheral CD4+ T cell counts and CD8+ T cell counts decreased in chronic HBV infection. In chronic hepatitis B patients, proportions of CD8+CD38+ T cells were $62.0\% \pm 14.7\%$, much higher than those of HBV carriers and healthy con-

trols. In the 13 hepatitis B patients who were treated and responded to adefovir dipivoxil, proportions of CD8+CD38+ T cells decreased from $53.9\% \pm 18.4\%$ pre-therapy to $20.1\% \pm 11.3\%$ by week 72 ($P < 0.001$), concomitant with viral load decline (HBV DNA fell from 7.31 to 3 log copies/mL). CD8+ T cell counts also underwent an average increase of 218 cells/ μ L by the end of 72-wk treatment. In those who failed the therapy, the CD8+CD38+ T cell population had more fluctuations.

CONCLUSION: CD8+ T cells abnormally activated in chronic HBV infection can be partially reversed by antiviral therapy. HBV-associated immune activation may be a crucial part of the pathogenesis and a promising target of treatment.

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Key words: Hepatitis B virus; Chronic hepatitis B; CD8+CD38+; T cell subsets

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health concern. Despite the availability of effective vaccines since the 1980s, approximately one third of the world's population has serological evidence of past or present infection with HBV^[1]. In China, over 8% of the

whole population is chronically infected. Although these patients can remain asymptomatic for years, chronic hepatitis B (CHB) is a progressive disease that can result in cirrhosis or hepatocellular carcinoma, and related death.

Hepatitis B virus is a noncytopathic virus. Clinical outcomes of HBV infection largely depend on the quality and strength of the host's immune response. Studies have revealed that T cellular immune responses are essential for disease pathogenesis^[2,3], and have identified CD8+ T lymphocytes as the main cellular subset responsible for viral control^[4,5]. Compared with acute self-limiting infection, lack of vigorous and multispecific T cell response in chronic HBV infection has been observed, which leads to the failure of viral clearance and the progression of disease^[6,7]. The composition of peripheral T cell subpopulations, on the other hand, serves as a valuable index for evaluating T immune status in chronic HBV infection. Impaired balance of peripheral T subpopulations has been reported at various stages of chronic HBV infections, associated with HBV replication levels, and can be partially restored after antiviral therapy^[8-11]. However, results from previous studies are controversial as regards the exact changes taking place during chronic HBV infection, and few have gone so far as to investigate the dynamics of more specific T cell subpopulations (e.g., CD38+/CD28+ activated T cell subpopulation or other functional subpopulations), or their changes upon antiviral treatment.

CD38, a marker of T cell activation, has shown elevated level of expression with many acute or chronic infections^[12,13]. With some infections, a correction of CD38 expression level could also be observed soon after effective treatment, e.g. human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) infections^[14]. Whether it is the same case with chronic HBV infection deserves more exploration. Adefovir dipivoxil (ADV), a synthetic nucleotide analogue of adenosine monophosphate, has been proven to provide biochemical, virological and histological improvement at the 10 mg oral dose daily^[15-17]. Its potency at inhibiting HBV replication raises the possibility that ADV may help to improve and restore the T cell profile, including CD38+ expression. The present investigation intended to give an initial assessment of the peripheral T lymphocyte subpopulations at different clinical stages of chronic HBV infection, and evaluate dynamics of these subpopulations with ADV monotherapy in treatment-naïve chronic hepatitis B patients, especially those of CD8+CD38+ T lymphocytes.

MATERIALS AND METHODS

Patients

A total of 60 patients were enrolled from March to November 2007 at the Outpatient Hepatitis Clinic of Peking Union Medical College Hospital (PUMCH), Beijing, China. Among them, 35 patients displayed clinical, biochemical and virological evidence of HBe-positive chronic hepatitis B [hepatitis B surface antigen (HBsAg) positive, hepatitis B e antigen (HBeAg) positive, anti-HBe negative, anti-HBc positive], with fluctuating levels

Table 1 Baseline characteristics of the patients (*n* = 95) *n* (%)

Characteristics	Hepatitis B patients (<i>n</i> = 35)	HBV carriers (<i>n</i> = 25)	Healthy controls (<i>n</i> = 35)
Mean age (yr)	34.4 ± 13.5	31.2 ± 13.3	34.0 ± 9.1
Sex (male/female)	29/6	13/12	21/14
Ethnic background	Han 35 (100)	Han 25 (100)	Han 35 (100)
Serum ALT level (IU/L)	281 ± 59.1	25.3 ± 11.5	21.4 ± 10.2
HBV DNA level (log copies/mL)	7.68 ± 0.98	8.43 ± 0.89	< 3
Positive for HBeAg	35 (100)	25 (100)	0 (0)
Prior medication for HBV	0 (0)	0 (0)	0 (0)

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

of alanine aminotransferase (ALT) (ranging from 82 to 252 U/L) and HBV DNA (ranging from 7.8×10^5 to 4.3×10^8 copies/mL). The other 25 patients were assigned as HBV carriers with positive HBsAg, various HBV DNA levels and normal ALTs. Standards of patient selection were based on 2009 AASLD (American Association for the Study of Liver Diseases) guidelines on chronic hepatitis B^[18]. Another 35 healthy subjects were also included as controls. All individuals were negative for anti-hepatitis C virus and HIV-1, and for other markers of viral or autoimmune hepatitis. The study protocol was approved by the local Ethical Committee, and all subjects provided written informed consent. Full baseline characteristics and virological data are summarized in Table 1.

Among the 35 chronic hepatitis B patients, 17 were then assigned to adefovir dipivoxil monotherapy at the oral dose of 10 mg/d (Deyang Huakang Pharmaceutical Co., Ltd., Sichuan, China), based on their clinical manifestations and patient agreement. All treated patients were followed for at least 48 wk after adefovir dipivoxil initiation; 14 of them were followed for 72 wk, and still remain on the follow-up list. Virological, biochemical and serological assessments, as well as T cell subset measurement of these patients, were carried out before treatment and at each follow-up point.

Virological assessments

Serum HBV DNA load was assessed with real-time fluorescent quantitative polymerase chain reaction method (Real-Time-PCR) using a Lightcycler PCR system (FQD-33A, Bioer) with a lowest limit of detection of approximately 10^3 viral copies/mL. The experimental procedures were performed in strict accordance with the reagent kit (Shenzheng PG Biotech Co., Ltd.) package insert. The primer was provided in the kit, the reaction volume was 40 μ L, and the reaction condition was 37°C for 5 min, 94°C for 1 min then 40 cycles as 95°C for 5 s and 60°C for 30 s. HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb) were measured by ELISA (enzyme-linked immunosorbent assay) method (Anthos 2010, Austria). The experimental methods followed the guidelines written in the reagent kit (Sino-American Biotech Co., Ltd.).

Table 2 Counts and proportions of patient peripheral lymphocyte subsets

T subsets	CHB patients (n = 35)	HBV carriers (n = 25)	Healthy controls (n = 35)
White blood cells $\times 10^9/L$	4.96 ± 1.18^b	5.60 ± 0.96^b	6.53 ± 1.42
Lymphocytes $\times 10^9/L$ (%)	1.60 ± 0.51^b (32.7 $\pm$ 8.7)	1.72 ± 0.44^b (31.2 $\pm$ 8.8)	2.13 ± 0.49 (33.1 $\pm$ 6.6)
Natural killer cells / μL (%)	197 ± 102^a (12.2 $\pm$ 5.1)	272 ± 189 (15.1 $\pm$ 8.4)	310 ± 181 (14.6 $\pm$ 7.8)
B cells/ μL (%)	228 ± 148 (14.3 $\pm$ 6.3)	220 ± 113 (12.6 $\pm$ 4.6)	242 ± 95 (11.4 $\pm$ 3.8)
CD3+ T cells/ μL (%)	1145 ± 380^b (71.5 $\pm$ 7.1)	1190 ± 278^b (70.4 $\pm$ 9.6)	1516 ± 382 (71.4 $\pm$ 7.6)
CD3+CD4+ T cells/ μL (%)	573 ± 194^b (36.3 $\pm$ 6.9)	673 ± 148^b (40.1 $\pm$ 6.9)	816 ± 259 (38.2 $\pm$ 7.0)
CD3+CD8+ T cells/ μL (%)	489 ± 213^a (30.1 $\pm$ 8.0)	445 ± 157^b (25.9 $\pm$ 6.1)	609 ± 177 (28.9 $\pm$ 5.7)
CD4+/CD8+	1.35 ± 0.62	1.64 ± 0.50	1.39 ± 0.44
CD8+CD38+ T cells/ μL (%)	301 ± 152 (62.0 $\pm$ 14.7 ^{b,d})	285 ± 121 (51.8 $\pm$ 18.5)	274 ± 81 (46.3 $\pm$ 11.9)
CD4+CD28+ T cells/ μL (%)	532 ± 191^b (96.0)	622 ± 149^a (95.7)	744 ± 221 (94.7)
CD8+CD28+ T cells/ μL (%)	235 ± 125^a (51.1 $\pm$ 15.5)	245 ± 100^a (55.7 $\pm$ 16.0)	306 ± 94 (51.3 $\pm$ 11.7)

^aMedians were used as the CD4+CD28+ T cell proportions were non-normal variables according to Kolmogorov-Smirnov Normality Test. ^a $P < 0.05$, ^b $P < 0.01$ vs normal healthy controls; ^d $P < 0.01$ vs hepatitis B virus (HBV) carriers. CHB: Chronic hepatitis B.

Peripheral blood T lymphocyte subset measurement

Peripheral blood T cell subset measurements were routinely performed during each follow-up review. Percentages of T cell subsets were determined on 100 μL ethylenediamine tetra-acetic acid (EDTA) blood sample, using a three-color direct immunofluorescence method (Beckman-Coulter, USA, EPICS2XL). In sample Tube 1, CD3+ T cells were autogated and analyzed for CD4 and CD8 cell expression using CD4/CD8/CD3 cell triple staining. In Tube 2, CD16/CD19/CD3 cell triple staining was used to identify B cells and natural killer (NK) cells. In Tubes 3, 4 and 5, CD38/CD8/CD3, CD28/CD8/CD3 and CD28/CD4/CD3 cell triple antibody cocktails were used, respectively, for analysis of CD38 and CD28 expression. Absolute counts of lymphocyte subpopulations were calculated according to complete blood cell counts on the same day.

Statistical analysis

SPSS version 11.0 was used. Baseline variables were assessed for all 95 subjects. For those who were included in the adefovir dipivoxil treatment and follow-up, virological and immunological parameters till the end of 72 wk were collected. Normal variables were summarized as means and standard deviations, and non-normal variables as medians and interquartile range (IQR) according to Kolmogorov-Smirnov Normality Test. Normal data were compared by Student *t* test or one-way ANOVA adjusted for multiple comparison, as appropriate. Multiple comparison of non-normal data was carried out by Kruskal Wallis test. All tests were two-sided, and a *P*-value ≤ 0.05 was considered significant. Associations between variables were assessed using Spearman's rank correlations. Tested results of serum HBV DNA which were below the lower limit of detection (less than 1000 copies per milliliter) were all analyzed as being 1000 copies per milliliter for the ease of analysis.

RESULTS

T lymphocyte subsets in chronic HBV infection

As shown in Table 2, the T lymphocyte counts in chronic

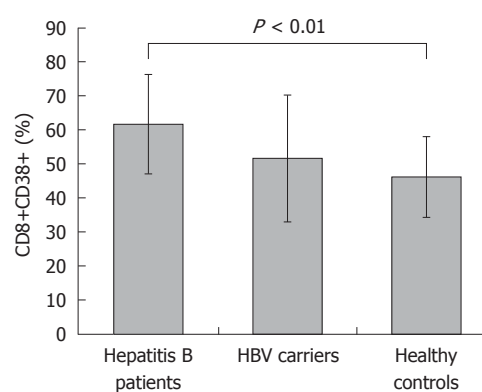


Figure 1 CD8+CD38+ T cell proportions in hepatitis B patients, hepatitis B virus carriers and healthy controls. Levels of CD8+CD38+ T cells in chronic hepatitis B patients were markedly higher than those of the other two groups. HBV: Hepatitis B virus.

hepatitis B patients and HBV carriers were 1145 ± 380 and 1190 ± 278 cells/ μL , respectively, both significantly lower than those of the healthy controls ($P < 0.01$). The absolute counts of CD4+ T cells and CD8+ T cells in chronic hepatitis B patients and HBV carriers were also lower than those of the controls. Yet the proportions thereof showed no significant differences between these three groups. The average counts of NK cells in chronic hepatitis B patients showed a moderate decrease compared to those of the carriers and controls. There were no significant differences in B cell counts between the different groups.

Levels of CD8+CD38+ T cells were also examined for each group of patients. We noticed a marked increase of CD8+CD38+ T cell proportions in chronic hepatitis B patients (62.0% $\pm$ 14.7%), compared to those of the HBV carriers (51.8% $\pm$ 18.5%) and healthy controls (46.3% $\pm$ 11.9%) ($P < 0.01$) (Figure 1). No such differences were observed in absolute CD8+CD38+ T counts between these patients. The CD8+CD38+ T cell levels of the HBV carriers and healthy controls showed no differences.

The counts of functional subsets CD4+CD28+ and CD8+CD28+ T cells were both markedly decreased in

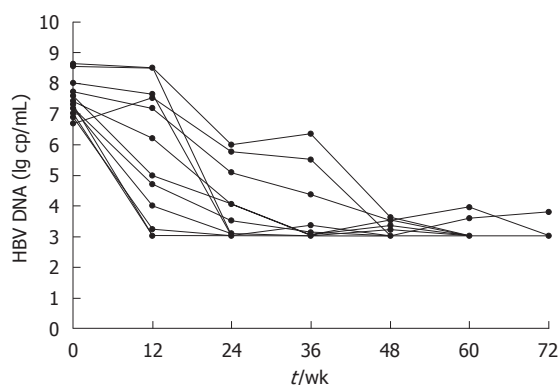


Figure 2 Hepatitis B viral load changes of the 13 patients who responded well to adefovir dipivoxil, by week of treatment. HBV: Hepatitis B virus.

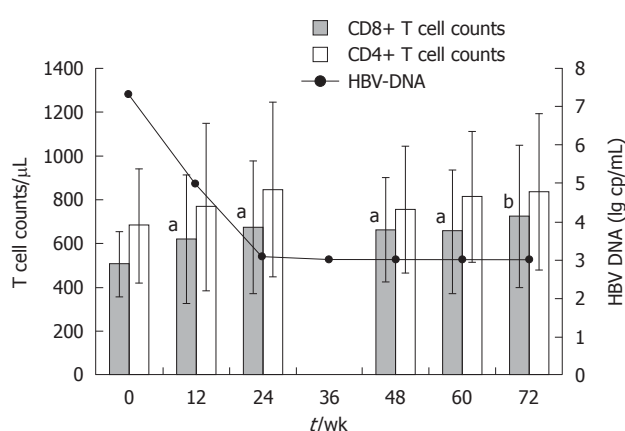


Figure 3 Changes of CD3+CD8+ and CD3+CD4+ T cell counts and median hepatitis B virus DNA in good responders, by week of adefovir dipivoxil treatment. ^a $P < 0.05$, ^b $P < 0.01$ vs baseline T cell counts before adefovir dipivoxil treatment. HBV: Hepatitis B virus.

chronic hepatitis B patients and HBV carriers. However, again, no significant differences were observed as to the proportions thereof compared with the normal controls.

Virological responses

Median change in serum HBV DNA load is presented as log₁₀ copies/mL. Among all the 17 patients receiving adefovir dipivoxil, 13 have achieved significant viral suppression, with their median HBV DNA decreased to undetectable levels ($< 10^3$ copies/mL) since 24 wk of treatment (Figure 2). However, the other 4 failed to respond to the therapy, either with no virological improvement or with a later rebound. As will be discussed later, changes in T lymphocyte subsets were analyzed based on patients' virological responses.

Changes in T lymphocyte subsets

CD3+CD8+ and CD3+CD4+ T lymphocyte subsets: As is shown in Figure 3, in those who responded well to adefovir dipivoxil, we observed a marked increase of 34.5% in absolute CD8+ T cell mean counts, from 505 ± 144 to 723 ± 324 cells/ μ L ($P < 0.01$) at the end of 72 wk of treatment. This increase began in the first

12 wk of treatment ($P < 0.05$), concomitant with the reduction of HBV DNA loads. Meanwhile, percentage of CD8+ T cells was also elevated at the end of 72 wk, from 30.7% to 33.2% ($P < 0.05$). In contrast, patients with poor response to adefovir dipivoxil showed no increase of CD8+ T cells.

Fluctuations of CD3+CD4+ T cell counts and proportions were also examined throughout the treatment. No significant changes were observed in either population. Absolute counts of CD4+ T cells at each time point are also shown in Figure 3.

As regards NK cells and B lymphocytes, neither the absolute numbers nor the proportions thereof showed any significant changes after the treatment.

CD8+CD38+ T cell subsets: In those who achieved good control of HBV viremia with adefovir dipivoxil, both the proportions and absolute counts of CD8+CD38+ T cells declined dramatically in peripheral blood (Figure 4A). From the first 12 wk after starting therapy, levels of CD8+CD38+ T cells began to decrease sharply. By week 24, the proportions of CD8+CD38+ T cells fell significantly from $53.9\% \pm 18.4\%$ to $36.8\% \pm 14.8\%$, while the absolute counts fell from 271 to 215 cells/ μ L ($P < 0.01$), and the same trends were sustained afterwards. By week 72, proportion of CD8+CD38+ T cell count had decreased as much as 62.8% from baseline to 20.1%. Absolute counts thereof showed a similar pattern of change. In contrast, the other 4 patients who did not achieve successful responses, though initially having some reduction of CD8+CD38+ T cell levels, showed CD8+CD38+ T cell fluctuations as their HBV load became unstable. CD8+CD38+ T cell changes of those who failed the therapy are depicted in Figure 4B.

As is apparent from Figure 4A, the drop in CD8+CD38+ T cell levels during the first 24 wk of treatment coincided with a marked change in serum HBV DNA load, with a median reduction from 7.30 log copies/mL to undetectable level. Regardless of virological responses to adefovir dipivoxil, in both patient groups the shape of the CD8+CD38+ T cell fluctuation curve followed that of the HBV DNA level. Also, there was a close relationship between changes in viral load and changes in CD8+CD38+ T cell levels. Further analysis from all available data paired for viral loads and T cell subsets indicated that CD8+CD38+ T cell subpopulations in chronic hepatitis B patients are significantly and positively related to serum HBV DNA log values (Figure 5, $r = 0.438$, $P = 0.001$).

Moreover, changes in CD8+CD38+ T cells were examined in 11 individuals who had achieved and maintained a viral load below 10^3 copies/mL, including at least two assessments of CD8+CD38+ T cells during the period of virological suppression. Of these 11 patients, 9 (82.2%) experienced a further fall in CD38+ T cell counts and proportions, whereas changes in HBV DNA were undetectable, all below 10^3 copies/mL. The mean change in CD8+CD38+/CD8+ percentage from

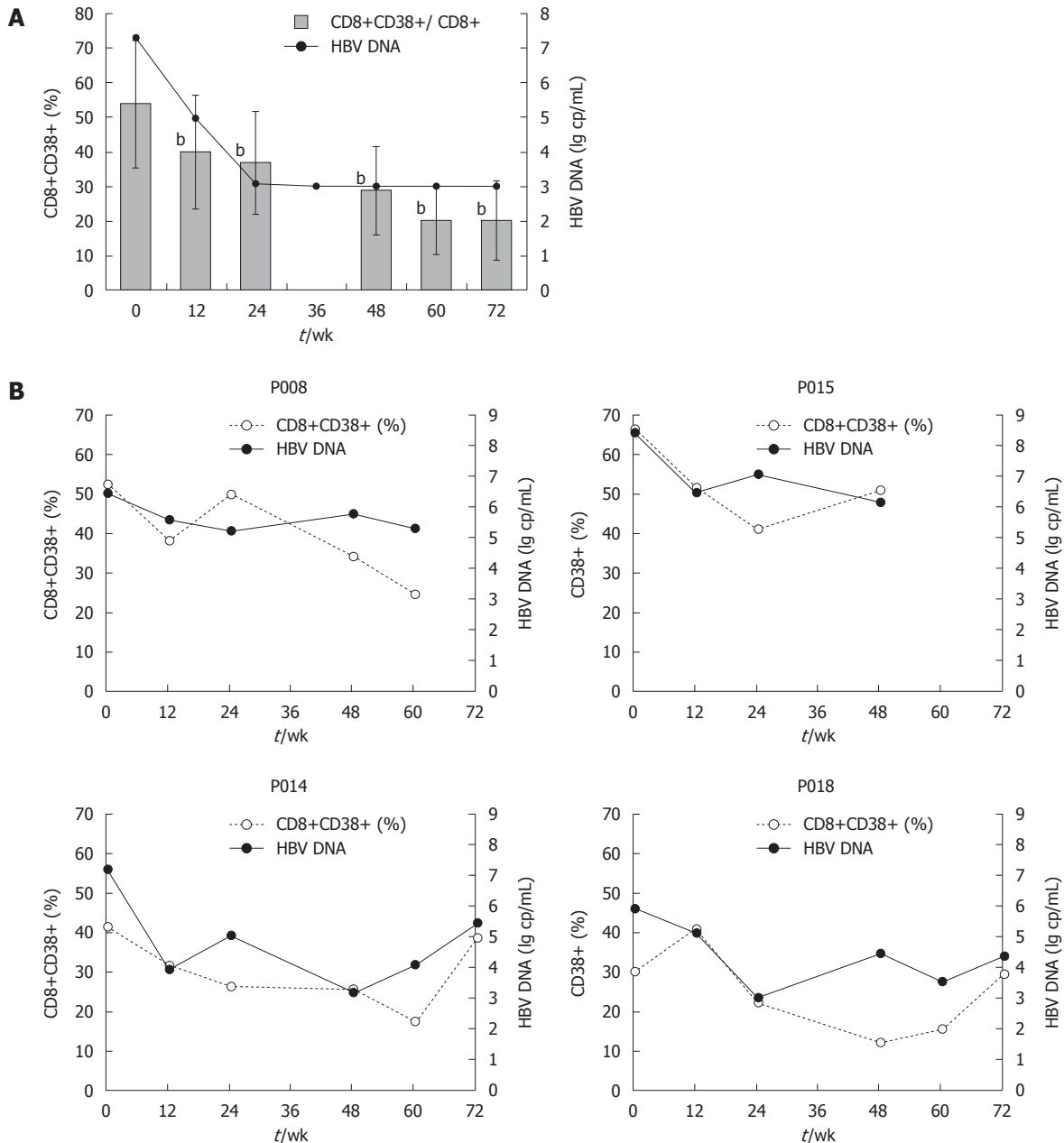


Figure 4 Changes of CD8+CD38+ T cell subsets and median hepatitis B virus DNA, by study week. A: Median CD8+CD38+ T cell proportions in good responders. ^b $P < 0.01$; B: Changes of CD8+CD38+ T cell subsets in 4 non-responding patients 008, 014, 015, and 018. Among them, Patient 008 and 015 only finished 60 and 48 wk of treatment, respectively. Patient 014 and 018 completed 72 wk of treatment. HBV: Hepatitis B virus.

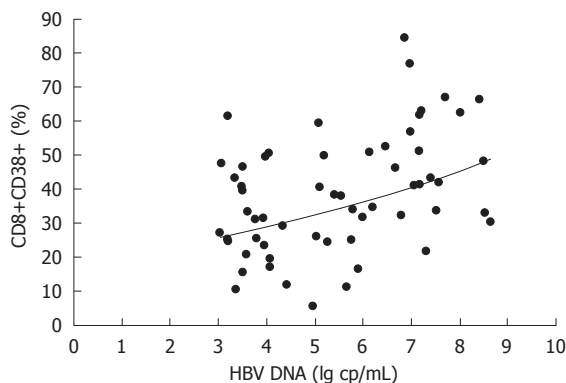


Figure 5 Correlations between CD8+CD38+ T cell levels and median hepatitis B virus DNA. HBV: Hepatitis B virus. $r = 0.438$, $P = 0.001$.

the first available count to the last was -10.6% ($P < 0.01$), indicating that reductions in CD8+CD38+ T cell proportions had continued even after serum HBV DNA become undetectable.

CD4+CD28+ and CD8+CD28+ T cell subsets: We also examined changes of CD28+ subpopulations both in CD8+ and CD4+ T lymphocytes before and after adefovir dipivoxil treatment. No statistically significant changes took place during the treatment (Data not shown).

DISCUSSION

In addition to HBV DNA level and liver function, chronic hepatitis B is further characterized by marked changes

in lymphocyte subpopulations and their activation status, which has only been poorly described in the literature. In the present study, we have identified discoordinate T cell profiles in chronic hepatitis B patients, with decreased counts of CD8+ T cells and robust CD8+ T activation, determined by an increase in the proportions of CD8+CD38+ T cells.

CD8+ and CD4+ T cells are two major components of the cellular immune system. As mentioned above, multiple studies have revealed that CD8+ T cells play an important role in clearance of the virus and progression of the disease. However, reports of CD8+ T cell subset changes in chronic hepatitis B patients have been rather conflicting. In the present study, we examined lymphocyte absolute counts as well as relative proportions in all 55 HBV infected patients and 35 healthy controls. We observed reductions of both CD8+ and CD4+ T cell levels in chronic hepatitis B patients and HBV carriers, which might reflect the T cell disturbance and suppression. Furthermore, in conjunction with the adefovir dipivoxil monotherapy, a marked elevation of CD8+ T cell levels took place, which demonstrated a partial restoration of T cell subsets and T cell immunity after the treatment. Prior studies have suggested that antiviral therapy can also overcome CD8+ T cell hypo-responsiveness in chronic HBV infection^[14,15]. We believed that there could be some improvement of T cell functions with adefovir dipivoxil treatment. However, right at this point, more functional studies are needed in order to make a conclusion. On the other hand, neither counts nor proportions of CD4+ T cells demonstrated any significant changes throughout the therapy in our study, despite the initial reduction of CD4+ T cells in chronically infected patients. However, some have suggested that functions of HBV specific CD4+ T cells would be improved in response to antiviral treatment^[19]. The peripheral CD4+/CD8+ ratios were much the same level in these three groups, perhaps because of the relatively localized infection and immunity that takes place in subjects with HBV infection. In addition, some prior studies have indicated that CD4+/CD8+ ratio of liver-derived lymphocytes, instead of peripheral lymphocytes, appeared to be more related to the level of HBV replication^[20]. In this sense, our analysis of the peripheral T cell composition had its limitations in directly evaluating the local immune functions. However, it is still a much easier way for evaluating the immune status in a more general clinical practice.

The further evaluation of each functional and activated T cell subset in chronic HBV infection highlighted the group of CD8+CD38+ activated T cell subsets. We have found much higher proportions of circulating CD8+CD38+ T cells in chronic hepatitis B patients. CD38 is a surface glycoprotein existing on many immune cells. As a marker of cell activation, it is associated with many infectious diseases such as HIV, EBV or other infections. In certain infections, CD8+CD38+ T cells undergo a rapid up-and-down pattern after the infection once the immune control of acute phase is achieved^[21].

However, persistency of immune activation and CD38+ expression throughout the acute and chronic phase is also possible, which may reflect the failure of the host's immune system to fully suppress viral replication. According to our observation of the peripheral CD8+CD38+ T cells in chronic hepatitis B patients, chronic HBV infection showed a mode of persistent T cell activation, just like that of HIV infection. However, elevations of CD8+CD38+ T cells in chronic hepatitis B patients were not so high as those of HIV-, EBV- or cytomegalovirus-infected patients^[5,6,13,22], perhaps due to the relatively localized infection of HBV in liver tissue in contrast to other systemic infections. On the other hand, HBV carriers, though also chronically infected, had similar levels of CD8+CD38+ T cells as healthy controls. This difference of CD8+ T activation may help to explain the relative immune quiescence of HBV carriers.

In further assessment of the 17 patients receiving adefovir dipivoxil monotherapy, we found that not only did the treatment effectively inhibit HBV replication, but it also resulted in a concomitant, profound and rapid decline in the CD8+CD38+ T cell levels. This reduction in CD8+CD38+ T cells began to take place after the first 12 weeks of treatment, and in the majority of treated patients, CD8+CD38+ T cell levels finally became fully normalized. This recovery of CD8+CD38+ T cell proportions confirmed the abnormal T cell activation as a result of the virological failure in active HBV infection. A further correlation analysis showed a positive and significant relationship between the CD8+CD38+ T cell proportions and serum HBV DNA, which indicated a T phenotype drift with viral stimulation. However, at this stage, it remains unclear whether the immune activation we observed was only a secondary change of viral insults, or part of HBV pathogenesis. Based on the variance of T cell activation between hepatitis B patients and HBV carriers with similar levels of viremia, CD8+CD38+ activated T cells most probably represent multiple interactions between viral and host factors. Studies of immune activation in HIV have suggested that CD8 T cell activation levels can predict the rate of disease progression independent of viral load, though the causes of this immune activation are likely multifactorial^[23,24]. Furthermore, some have tried to target HIV-associated immune activation by using immunomodulatory agents in addition to antiretroviral therapy^[25-27]. The role of immune activation in chronic HBV infection has been poorly described. Here, our study of CD8+CD38+ activated T cells raises the possibility that T immune activation helps to shape the fate of chronic HBV infection and, therefore, use of immunomodulatory agents may help with the control of hepatitis B virus. However, a larger sample size and more specific and functional studies are needed to establish this conclusion.

Moreover, a continued decline in CD8+CD38+ T cell levels was observed even after HBV DNA became undetectable and CD8+CD38+ T cells became normalized in successfully treated patients. This finding is interesting,

because we have also observed similar phenomena in other treated viral infections. It could be an indication of presentations of the residual virus below the lower limit of viremia detection. Another possible explanation is that the CD8+CD38+ T cell levels under normal conditions may actually present an overall balance between the human immune system and occasional environmental insults, and that non-specific antiviral medications such as adefovir dipivoxil may also have an effect on these other pathogens, thus reducing the chances of immune activation in these patients. A longer follow-up of these patients during and after treatment may help to better explain the dynamics of the CD8+CD38+ T cell subsets.

The functional subsets of CD8+CD28+ and CD4+CD28+ T cells were also examined in our study. CD28+ acts as a co-stimulating molecule on the surface of T cells. However, according to a few currently available evaluations^[12,28], unlike HIV infection which leads to the down-regulation of CD28 on the surface of T cells^[29,30], HBV infection seems to have no significant impact on CD28 expression. In our study, the proportions of CD8+CD28+ and CD4+CD28+ T cells in the three groups were similar to each other. The absolute counts, though lower in chronic hepatitis patients and HBV carriers, were actually reflections of the decreased CD8+ and CD4+ T cell levels. Further treatment, which resulted in good control of the virus, did not change CD28 expression on the CD4+ or CD8+ T cells either, which further confirmed our observations before the treatment.

COMMENTS

Background

Chronic Hepatitis B virus (HBV) infection is a global health concern. Studies have identified that T cellular immune responses are essential for disease control. However, profiles of T cell subsets in chronic HBV infection, especially those of activated T cell subsets, have not been fully revealed. The authors studied the peripheral T cell subsets in chronic HBV infection, and their dynamics in response to adefovir dipivoxil monotherapy.

Research frontiers

T cellular immune responses are essential for pathogenesis of chronic HBV infection; impaired balance of peripheral T subpopulations has been reported at various stages of chronic HBV infections, and can be partially restored after antiviral therapy.

Innovations and breakthroughs

The study systemically assessed the peripheral T lymphocyte subsets in different types of chronic HBV infection. For the first time, the authors highlighted the group of CD8+CD38+ activated T cells, and their dynamics before and after treatment. The authors found T cell activation was linked with active HBV infection, and declined with successful antiviral treatment, which suggested the crucial role of immune activation in HBV pathogenesis.

Applications

The authors clarified differences of peripheral T cell subsets between different clinical types of chronic HBV infection, and changes thereof in response to treatment. HBV-associated immune activation may be crucial for the pathogenesis of HBV infection. This adds to current knowledge of T immune functions in chronic HBV infection, and helps with a better understanding of HBV pathogenesis. The abnormal T immune activation status in chronic hepatitis B suggests the possible use of immunomodulatory agents as further treatment.

Peer review

This is an interesting and well-written manuscript that clearly shows the link between CD8+38+ T-cells and HBV DNA levels. The authors described a decrease in total CD4 and CD8 T cell counts in chronic hepatitis B along with a

higher proportion of CD8/CD38 cells after treatment. The amount of activated cells decreased in patients responding to an antiviral therapy while the amount of CD8 cells rose. The authors suggest HBV-associated immune activation may be a crucial part of the pathogenesis and a promising target of treatment.

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Hepatotropic growth factors protect hepatocytes during inflammation by upregulation of antioxidative systems

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Abstract

AIM: To investigate effects of hepatotropic growth factors on radical production in rat hepatocytes during sepsis.

METHODS: Rat hepatocytes, isolated by collagenase perfusion, were incubated with a lipopolysaccharide (LPS)-containing cytokine mixture of interleukin-1 β , tumor necrosis factor- α and interferon- γ to simulate sepsis and either co-incubated or pre-incubated with hepatotropic growth factors, e.g. hepatocyte growth factor, epidermal growth factor and/or transforming growth factor- α . Cells were analyzed for glutathione levels. Culture supernatants were assayed for produc-

tion of reactive oxygen intermediates (ROIs) as well as NO $_2^-$, NO $_3^-$ and S-nitrosothiols. To determine cellular damage, release of aspartate aminotransferase (AST) into the culture medium was analyzed. Activation of nuclear factor (NF)- κ B was measured by electrophoretic mobility shift assay.

RESULTS: Rat hepatocytes treated with the LPS-containing cytokine mixture showed a significant increase in ROI and nitrogen oxide intermediate formation. AST leakage was not significantly increased in cells treated with the LPS-containing cytokine mixture, independent of growth-factor co-stimulation. However, pretreatment with growth factors significantly reduced AST leakage and ROI formation while increasing cellular glutathione. Application of growth factors did not result in increased NF- κ B activation. Pretreatment with growth factors further increased formation of NO $_2^-$, NO $_3^-$ and S-nitrosothiols in hepatocytes stimulated with LPS-containing cytokine mixture. Thus, we propose that, together with an increase in glutathione increased NO $_2^-$, NO $_3^-$ formation might shift their metabolism towards non-toxic products.

CONCLUSION: Our data suggest that hepatotropic growth factors positively influence sepsis-induced hepatocellular injury by reducing cytotoxic ROI formation *via* induction of the cellular protective antioxidative systems.

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Key words: Primary human hepatocytes; Hepatocyte proliferation; Cytokines; Hepatotropic growth factors; Nitric oxide; Glutathione

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INTRODUCTION

After partial hepatectomy, the remaining liver tissue undergoes rapid regeneration of its lost mass. Although it has been studied for many years, the exact mechanisms and interactions of this regenerative process are still the focus of many investigations^[1-4]. Despite advances in surgical techniques and perioperative management, liver failure occasionally occurs after extended hepatectomy often being associated with postoperative infections that lead to multiple organ failure and death^[5,6].

Although a two-thirds resection of the liver is not fatal, there is increased sensitivity to endotoxin, caused by up-regulation of the toll-like receptor 4, in the period following experimental hepatectomy^[7]. Thus, intravenous injection of a sub-lethal dose of lipopolysaccharide (LPS) 48 h after surgery results in a high mortality in rats^[8]. LPS directly activates Kupffer cells (the hepatic macrophages) to produce the tumor necrosis factor (TNF)- α and other inflammatory cytokines^[9] through activation of the transcription factor, nuclear factor (NF)- κ B. During liver regeneration, however, cytokines as well as hepatotropic growth factors have been well demonstrated to be involved in the process of tissue regeneration^[10].

Numerous publications suggest a direct link between nitric oxide (NO) production, cellular loss of glutathione (GSH) and reduction of glutathione reductase activity. Thus, depletion of GSH reduces cellular NO levels while increasing superoxide formation, because GSH is an important cofactor for NO synthase^[11-16]. Togo *et al.*^[17] suggest that NF- κ B is the major transcription factor regulating the initial steps of liver regeneration. Growth factors, by different mechanisms, play an essential role in cell growth, proliferation, differentiation and DNA synthesis^[18-21]. Certain interplays between cytokines and growth factors indeed seem to exist. Inflammatory cytokines increase the intracellular radical formation if not being blocked by intracellular antioxidative systems, e.g. GSH^[22]. Therefore, it might be possible that adequate proliferation and regeneration occurs after partial hepatectomy, and the interplay of growth factors and cytokines could be shifted towards protective proliferation rather than hepatocellular injury.

Using an experimental model of sepsis/inflammation, we investigated the effects of hepatotropic growth factors, hepatocyte growth factor (HGF), epidermal growth factor (EGF) and/or transforming growth factor (TGF)- α on radical production and glutathione content in rat hepatocytes that were exposed to an inflammatory cytokine mixture of interferon (IFN)- γ , TNF- α and interleukin

(IL)-1 β , including LPS.

MATERIALS AND METHODS

Isolation, culture and treatment of primary rat hepatocytes

Rat hepatocytes were isolated from healthy Sprague-Dawley rats with a body weight between 250 and 300 g (Fa. Harlan-Winkelmann, Borcheln, Germany) in accordance with the institutional guidelines of the Charité (Berlin, Germany) by collagenase P (Boehringer, Mannheim, Germany) digestion as described previously^[23]. Hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50 *g*. Cells were further purified by density gradient centrifugation using 30% Percoll (Pharmacia, Piscataway, NJ, USA). Hepatocyte purity, assessed by microscopy, was > 95% and viability, examined by trypan blue exclusion method, was consistently > 90%. Immediately after isolation, hepatocytes were plated onto gelatin-coated culture dishes (5×10^4 cells/cm²) in Williams medium E (0.5 mmol/L L-arginine, 1 μ mol/L insulin, 15 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% fetal calf serum). The next day, experiments were performed in serum-free medium. To imitate inflammation, cells were stimulated with a cytokine mixture (CM) consisting of 100 U/mL IFN- γ , 500 U/mL TNF- α , 10 U/mL IL-1 β and 10 μ g/mL LPS (*Escherichia coli* 111:B4) for 24 h. To investigate the effect of growth factors on inflammation, cells were either co-stimulated or pretreated (12 h) with 20 ng/mL HGF, 30 ng/mL EGF and/or 20 ng/mL TGF- α .

Measurement of NO₂⁻, NO₃⁻ and S-nitrosothiols

Culture supernatants were assayed for the stable end products of NO oxidation (NO₂⁻ and NO₃⁻) and S-nitrosothiols using modified procedures based on the Griess reaction as described previously^[24].

Aspartate aminotransferase measurement

In order to evaluate cellular damage, culture supernatants were measured for aspartate aminotransferase (AST) leakage using commercially available reaction kits (Roche Diagnostics, Mannheim, Germany).

Determination of cellular GSH levels

To evaluate total cellular GSH levels [GSH + oxidized glutathione (GSSG)] cells were suspended in 1 mL metaphosphoric acid (3%) and centrifuged at 1000 *g* for 5 min. Supernatants were adjusted to pH 7.5-8.0 with K₂CO₃. Total cellular GSH was assayed, using an enzymatic recycling procedure, as described previously^[22]. Reduced GSH was sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic-acid) (DTNB) to GSSG. The rate of DTNB formation was monitored at 412 nm and glutathione content was determined from a standard curve. To determine GSSG, GSH was masked with 2-vinylpyridine. Then, GSSG was reduced by NADPH to GSH in the presence of glutathione reductase to react again with DTNB. Oxidized and

reduced GSH that may be released in the supernatant were measured in the same way. All data were normalized to total protein content determined from cell pellets.

Determination of superoxide (O_2^-)

Release of O_2^- into culture supernatant was measured by monitoring the superoxide dismutase-dependent reduction of 160 $\mu\text{mol/L}$ ferricytochrome c at 550 nm and 37°C, where 1 mol O_2^- reduces 1 mol ferricytochrome c^[14].

Measurement of NF- κ B activation

The NF- κ B binding activity was analyzed by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from cells that were homogenized in sucrose buffer (2.1 mol/L sucrose, 10 mmol/L HEPES, 1 mmol/L MgCl_2 , 5 mmol/L NaF, 0.5 mmol/L Leupeptin, 0.5 mmol/L pepstatin, 5 mmol/L aprotinin, 1 mmol/L DTT, and 0.1 mmol/L PMSF). Nuclei were separated by centrifugation (35 000 g, 3 h, 4°C) and washed twice in sucrose washing buffer. Nuclei were resuspended in high-salt buffer (20 mmol/L HEPES at pH 7.9, 1.5 mmol/L MgCl_2 , 440 mmol/L NaCl, 0.2 mmol/L EDTA, 25% glycerol, 5 mmol/L NaF, 0.1 mmol/L PMSF, 0.5 mmol/L leupeptin, 0.5 mmol/L pepstatin, 5 mmol/L aprotinin, and 1 mmol/L DTT). After incubation on ice for 50 min, nuclei were spun down (14 000 g, 15 min, 4°C). Following quantification, protein extracts were stored at -70°C. NF- κ B binding activity was performed as described previously^[25]. The DNA probe used for EMSA corresponded to the high-affinity kB sequence found in the mouse κ light chain enhancer. Two oligonucleotides (sense 5'-AGCTTGGGGACTTTCCTACTAGTACG-3', antisense 5'-AATTCGTACTAGTGGAAAGTCCCCA-3') were annealed to generate a double-stranded probe. Labeling was accomplished by the Klenow fragment of DNA polymerase I in the presence of dGTP, dCTP, dTTP and α [³²P] dATP. After labeling, the probe was added to 5 μg nuclear protein and 5 μg poly-dI-dC (Pharmacia Biotech Enzyme GmbH, Freiburg, Germany). Binding reactions were carried out in 10 mmol/L Tris-HCl (100 mmol/L NaCl, and 4% glycerol, pH 7.5) for 30 min on ice. DNA-protein complexes were resolved by electrophoresis in a 4% non-denaturing polyacrylamide gel. Monoclonal antibodies raised against various NF- κ B subunits (p50, p52, rel A/p65, C rel, and rel B; Santa Cruz Biotechnology, Heidelberg, Germany) were used to confirm the nature of the DNA-protein complex. Competition assay was performed using unlabeled κ B probe in 10- 50- and 100-fold concentrations^[25].

Statistical analysis

Results are expressed as mean $\pm$ SE of at least five independent experiments ($N = 5$) measured in triplicates ($n = 3$). Data sets were compared by Kruskal-Wallis followed by Dunn's multiple comparison test (GraphPad Prism software; El Camino Real, Sunnyvale, CA USA). $P < 0.05$ was taken as minimum level of significance.

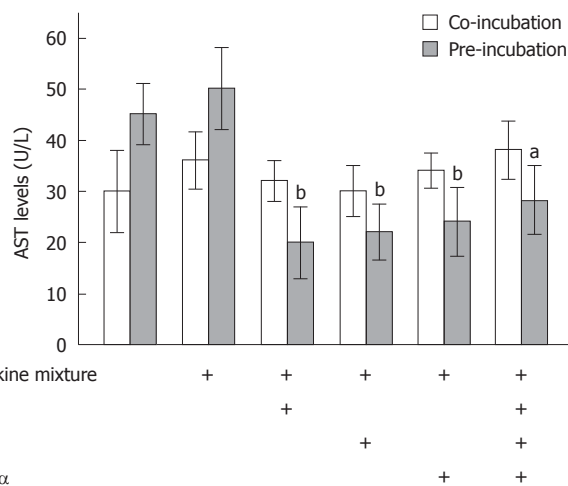


Figure 1 Pre-stimulation with growth factors significantly reduces cellular damage induced by lipopolysaccharide-containing cytokine mixture. Primary rat hepatocytes ($N = 5$, $n = 3$) treated with lipopolysaccharide (LPS)-containing cytokine mixture (CM) for 24 h showed a slight increase in aspartate aminotransferase (AST) leakage into the culture supernatant. Co-incubation with the hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor (TGF)- α , individually or in combination, did not reduce AST leakage significantly (empty bars). However, preincubation with these hepatotropic growth factors resulted in a significant reduction in AST leakage (grey bars). * $P < 0.05$, ^b $P < 0.005$ vs corresponding rat hepatocytes treated with LPS-containing CM alone.

RESULTS

Measurement of AST leakage in rat hepatocytes pre- and co-treated with growth factors

Incubation of primary rat hepatocytes ($N = 5$, $n = 3$) with LPS-containing CM led to a slight increase in AST levels in the culture supernatant (30.0 ± 8.0 to 36.0 ± 5.6 U/L and 45.0 ± 6.0 to 50.0 ± 8.0 U/L). Co-incubation with growth factors (20 ng/mL HGF, 30 ng/mL EGF and/or 20 ng/mL TGF- α) individually or in combination did not reduce AST leakage (Figure 1, empty bars). However, preincubation with growth factors significantly reduced AST leakage in cells treated with LPS-containing CM (Figure 1, grey bars).

Determination of reactive oxygen intermediate levels in rat hepatocytes co-treated with growth factors

Incubation of primary rat hepatocytes ($N = 5$, $n = 3$) with the LPS-containing CM slightly increased reactive oxygen intermediate (ROI) production (63.0 ± 6.7 to 80.0 ± 9.0 pmol O_2^- /min every 10^5 cells), while the intracellular glutathione levels were not notably affected as compared to untreated controls. Co-incubation with all growth factors combined slightly reduced ROI levels (Figure 2A and B, empty bars).

Determination of cellular GSH from rat hepatocytes pretreated with growth factors

Rat hepatocytes ($N = 5$, $n = 3$) were pretreated with growth factors 12 h prior to incubation with LPS-containing CM. As observed before, LPS-containing CM

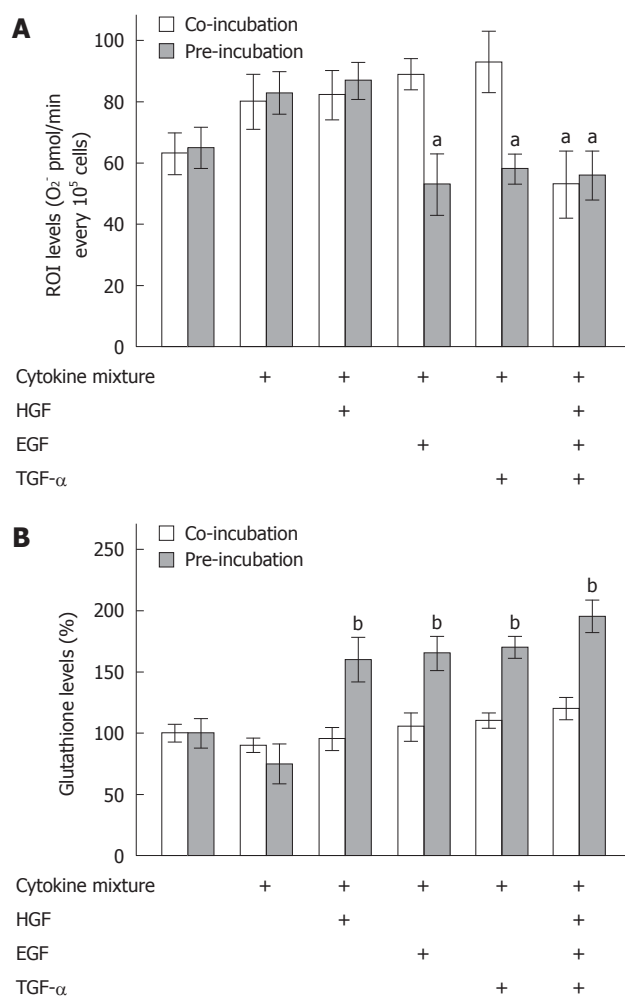


Figure 2 Increased oxidative stress in rat hepatocytes treated with lipopolysaccharide-containing cytokine mixture. A: Treatment of primary rat hepatocytes ($N = 5$, $n = 3$) with lipopolysaccharide (LPS)-containing cytokine mixture (CM) for 24 h caused a slight increase in O₂⁻ production. B: Cellular glutathione levels were not significantly affected by this treatment. Co-incubation with single hepatocyte growth factor (HGF), epidermal growth factor (EGF) or transforming growth factor (TGF)- α did not reduce reactive oxygen intermediate (ROI) production significantly. Co-incubation with the hepatotropic growth factor mixture alone was able to reduce ROI production significantly. Furthermore, co-incubation with the growth factors did not alter cellular glutathione levels (empty bars). On the other hand, preincubation with these growth factors, individually or in combination, significantly reduced ROI production (except for pretreatment with HGF alone). Preincubation with the different growth factors increased cellular glutathione significantly (grey bars). ^a $P < 0.05$, ^b $P < 0.001$ vs corresponding rat hepatocytes treated with LPS-containing CM alone.

slightly increased ROI production (65.0 ± 6.7 to 70.0 ± 7.0 pmol O₂⁻/min every 10⁵ cells) without a notable effect on intracellular glutathione levels (Figure 2A and B, grey bars). However, pretreatment with growth factors, both individually or in combination, significantly reduced ROI production by subsequent stimulation with LPS-containing CM. At the same time, intracellular glutathione levels were significantly increased. This goes along with the reduction in AST leakage observed with growth-factor-pretreated cells (Figure 1, grey bars). Combination of all three growth factors did not further decrease ROI production or increase intracellular glutathione compared to

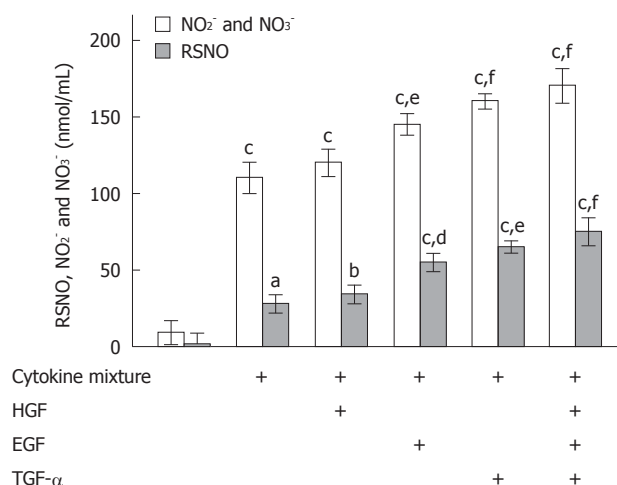


Figure 3 Hepatotrophic growth factors increase nitric oxide formation in primary rat hepatocytes. NO₂⁻ plus NO₃⁻ (empty bars) and RSNO (grey bars) levels demonstrated a significant increase after hepatocyte ($N = 5$, $n = 3$) pretreatment with growth factors and subsequent stimulation with lipopolysaccharide (LPS)-containing cytokine mixture (CM), if compared to treatment with LPS-containing CM alone [except for pretreatment with hepatocyte growth factor (HGF) alone]. ^a $P < 0.05$, ^b $P < 0.005$, ^c $P < 0.001$ vs corresponding untreated rat hepatocytes; ^d $P < 0.05$, ^e $P < 0.005$, ^f $P < 0.001$ vs corresponding rat hepatocytes treated with LPS-containing CM alone. NO: Nitric oxide; EGF: Epidermal growth factor; TGF: Transforming growth factor.

treatment with single growth factors. Pretreatment with HGF alone was not able to reduce ROI production by subsequent stimulation with LPS-containing CM.

Determination of NO₂⁻, NO₃⁻ and S-nitrosothiol formation in rat hepatocytes pretreated with growth factors

Incubation of hepatocytes ($N = 5$, $n = 3$) with the LPS-containing CM led to a significant increase in NO production as compared to untreated controls. Formation of stable end products of NO oxidation (NO₂⁻ and NO₃⁻) and S-nitrosothiols was even more increased in hepatocytes pretreated with growth factors when subsequently stimulated with LPS-containing CM. Pretreatment with HGF alone did not further increase NO₂⁻, NO₃⁻ and S-nitrosothiols compared to stimulated cells without pretreatment (Figure 3). This was in accordance with the lack of reduction of ROIs under the same conditions.

Determination of NF- κ B activation in rat hepatocytes stimulated with LPS-containing CM pretreated with or without growth factors

Rat hepatocytes, with and without pretreatment with growth factors, were stimulated with LPS-containing CM. NF- κ B activation was measured at 0.5, 1, 2, 3 and 6 h after stimulation by EMSA. NF- κ B was markedly increased 6 h after stimulation with LPS-containing CM (Figure 4A). Pretreatment with the combined or individual growth factors did not further increase NF- κ B activation (Figure 4B). Moreover, growth factors alone (without LPS-containing CM) were not able to cause NF- κ B expression (Figure 4B). The competition assay

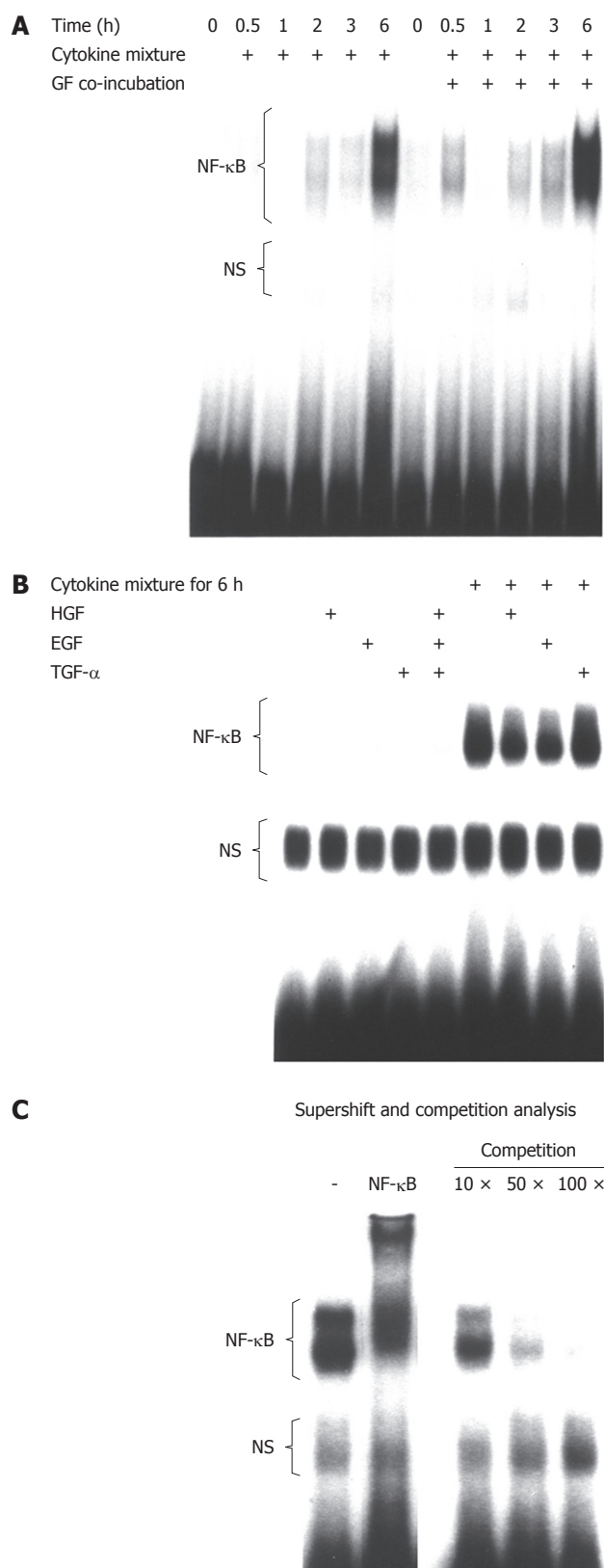


Figure 4 No additional nuclear factor- κ B activation by hepatotrophic growth factors. Electrophoretic mobility shift assay for nuclear factor (NF)- κ B after hepatocyte pretreatment with hepatotrophic growth factors and subsequent stimulation with lipopolysaccharide (LPS)-containing cytokine mixture (CM) demonstrated that stimulation with growth factors combined (A) or individually (B) did not further increase NF- κ B expression when compared to stimulation with LPS-containing CM alone. Super shift and competition analysis (C) of NF- κ B. HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor.

using an excess of unlabeled κ B probes demonstrated the specificity of the signal (Figure 4C).

DISCUSSION

Recovery after partial hepatectomy requires an adequate interplay between hepatotrophic growth factors and cytokines, as both factors are markedly involved and obviously well-balanced in the process of residual liver tissue proliferation and regeneration^[26,27]. In this context, it has been reported that IL-6 plays a crucial role for regeneration, because it is supposed to prime remnant hepatocytes, in a way that they can fully respond to growth factors and enter a pre-replicative phase (G1)^[26-28]. However, in our earlier studies, we have found that addition of IL-6 to hepatocyte cultures does not alter ROI or nitrogen oxide intermediate production in the presence of other inflammatory cytokines. When using the mentioned growth factors, there was also a lack of significant alterations in ROIs, and intracellular glutathione was seen. This suggests that growth factors have no direct impact on radical formation, cellular injury and/or cellular antioxidative protection systems.

Under septic or inflammatory conditions, as in the case of any infectious post-operative complication, when both plasma HGF and inflammatory cytokine levels are increased^[29-31], cytokine and growth factor compositions might be different. Indeed, increased cytokine levels and protein-protein interactions may have positive and negative effects on liver regeneration^[32,33]. Thus, IL-1 β is markedly expressed during inflammation, and acts as a very potent inhibitor of hepatocyte proliferation^[34]. Clinically observed, severe infections may seriously affect the post-operative course after liver resection, which results in an increased incidence of liver insufficiency and patient loss^[6,35,36].

Obviously, cytokines and growth factors act in a well-balanced process under normal regenerative conditions. To gain a better understanding of the avoidance of the deleterious effects of postoperative infectious complications following liver resection, the interplay of growth factors and cytokines was a focus of our attention.

As cytokine reduction is hard to achieve if inflammation has already occurred, we focused our analysis on the effects of hepatotrophic growth factor (pre)treatment in hepatocytes exposed to an inflammatory LPS-containing CM.

In the present study, we could demonstrate that growth factors, namely HGF, EGF and/or TGF- α may positively influence cytokine-induced hepatocellular injury. In pre-treated hepatocytes, we found increased NO levels, while the expression of NF- κ B was comparable to untreated controls. Our results confirm the study of Kaido *et al.*^[37] who have reported on successful prevention of post-operative liver failure in cirrhotic rats by continuous HGF supply. They have shown that rats with HGF-secreting fibroblasts (genetically modified to secrete rat HGF and implanted in syngeneic rat spleen 7 d prior to exposition exposure of to hepatotoxins)

showed a dramatic resistance to carbon tetrachloride- and LPS-induced liver injury, which resulted in a significantly improved survival rate (80% *vs* 20%). In the same line of evidence, Kosai *et al.*^[38] have shown that HGF treatment 6 h and 30 min before and 3 h after intra-peritoneal LPS administration resulted in a significant increase of survival in mice (75% *vs* 0%). Although not focusing on pathophysiological interactions of HGF and cytokines, they clearly described HGF-related hepatic protection in case of severe endotoxemia^[37,38].

Although several mechanisms may lead to hepatocyte injury, oxidative stress with increased radical formation as a consequence of inflammation, sepsis or ischemia-reperfusion, plays an important role. Intracellular antioxidative systems, e.g. p38-mitogen activated protein kinase or p21 may protect the cells, but they also decrease the hepatocyte proliferation rate by inhibiting hepatic DNA synthesis during the late G1 phase^[39,40]. Other intracellular antioxidative systems include upregulation of enzymes e.g. heme oxygenase-1 by NF- κ B^[41]. We hypothesize that increased glutathione synthesis reduces the amount of cytotoxic radical formation. As further mechanisms improve oxygen supply, subsequent NO-dependent vasodilatation may contribute to the growth-factor-related protection of rat hepatocytes during sepsis. This could explain the results of Seto *et al.*^[42] who have observed that HGF pretreatment attenuates LPS-induced sinusoidal endothelial cell injury and intra-sinusoidal fibrin deposition.

However, further studies are required because this kind of cell protection was present only in hepatocyte pretreatment. Indeed, direct stimulation of rat hepatocytes with growth factors had no impact on intracellular ROI levels, glutathione content or AST levels under septic conditions.

Nevertheless, this aspect could provide new therapeutic options in case of partial hepatectomy. Pretreatment with hepatotropic growth factors may potentially decrease the incidence of postoperative liver insufficiency in patients undergoing extended liver resection, and subsequent infectious complications by shifting the postoperative course towards growth-factor-related liver tissue proliferation rather than cytokine-related cellular injury.

COMMENTS

Background

The exact mechanisms and interactions of the regenerative process in the liver after partial hepatectomy remain unclear. The well-balanced interplay of liver growth factors and cytokines is strongly interfered when any infectious postoperative complications occur. This effect leads to higher mortality via radical formation.

Research frontiers

The deleterious effects of postoperative infectious complications following liver resection have not been examined adequately. In particular, the interplay of pretreated growth factors and cytokines was studied.

Innovations and breakthroughs

The main reason for increased survival of growth-factor-pre-treated hepatocytes is the intracellular antioxidative system that prevents cell-damaging radical formation. Nitric oxide production during sepsis especially increases cell survival.

Applications

Pretreatment with hepatotropic growth factors can be a new therapeutic option

in case of patients undergoing extended liver resection and may potentially decrease the incidence of postoperative liver insufficiency.

Terminology

Partial hepatectomy describes the process by which tumors are surgically removed from the liver. Cytokines are regulatory proteins that are released by cells of the immune system and act as mediators in the generation of an immune response.

Peer review

This paper is interesting and reports a large number of experiments. The methodology is well described and the results are clearly shown.

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Value of transient elastography for the prediction of variceal bleeding

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Abstract

AIM: To determine if liver stiffness (LS) measurements by means of transient elastography (TE) correlate with the presence of significant esophageal varices (EV) and if they can predict the occurrence of variceal bleeding.

METHODS: We studied 1000 cases of liver cirrhosis divided into 2 groups: patients without EV or with grade 1 varices (647 cases) and patients with significant varices (grade 2 and 3 EV) (353 cases). We divided the group of 540 cases with EV into another 2 subgroups: without variceal hemorrhage (375 patients) and patients with a history of variceal bleeding (165 cases). We compared the LS values between the groups using the unpaired t-test and we established cut-off LS values for the presence of significant EV and for the risk of bleeding by using the ROC curve.

RESULTS: The mean LS values in the 647 patients without or with grade 1 EV was statistically significantly lower than in the 353 patients with significant EV (26.29 ± 0.60 kPa vs 45.21 ± 1.07 kPa, $P < 0.0001$). Using the ROC curve we established a cut-off value of 31 kPa for the presence of EV, with 83% sensitivity (95%

CI: 79.73%-85.93%) and 62% specificity (95% CI: 57.15%-66.81%), with 76.2% positive predictive value (PPV) (95% CI: 72.72%-79.43%) and 71.3% negative predictive value (NPV) (95% CI: 66.37%-76.05%) (AUROC 0.7807, $P < 0.0001$). The mean LS values in the group with a history of variceal bleeding (165 patients) was statistically significantly higher than in the group with no bleeding history (375 patients): 51.92 ± 1.56 kPa vs 35.20 ± 0.91 kPa, $P < 0.0001$). For a cut-off value of 50.7 kPa, LS had 53.33% sensitivity (95% CI: 45.42%-61.13%) and 82.67% specificity (95% CI: 78.45%-86.36%), with 82.71% PPV (95% CI: 78.5%-86.4%) and 53.66% NPV (95% CI: 45.72%-61.47%) (AUROC 0.7300, $P < 0.0001$) for the prediction of esophageal bleeding.

CONCLUSION: LS measurement by means of TE is a reliable noninvasive method for the detection of EV and for the prediction of variceal bleeding.

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Key words: Liver stiffness; Transient elastography; Esophageal varices; Variceal bleeding

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INTRODUCTION

Transient elastography (TE) is a new promising noninva-

sive and rapid method for the diagnosis and quantification of liver fibrosis in patients with chronic liver disease. It was originally developed to detect solid malignancies in soft tissues such as breast cancer and prostate cancer^[1]. Liver stiffness (LS) measurement using TE is reproducible and independent of the operator^[2]. Some recent extensive studies have demonstrated that LS measurement with TE is a good alternative for liver biopsy. The amount of fibrosis can be quantified very easily and reliably and is feasible in more than 95% of the patients^[3-5]. In cirrhotic patients, LS measurements range from 12.5 to 75.5 kPa. However, the clinical relevance of these values is unknown.

The risk of variceal hemorrhage is clearly related to the size of esophageal varices (EV). Therefore primary prevention of variceal bleeding applies in patients with previously diagnosed large EV (grade 2 or 3) detected by periodical upper digestive endoscopy [Baveno V and American Association for the Study of Liver Diseases (AASLD) Consensus]^[6,7]. A generalized program of periodical upper tract endoscopy in these patients might result in a heavy economic burden even for developed countries. Furthermore, repeated examinations, when not performed under profound sedation, are often poorly accepted by patients who may refuse further follow up.

TE may be used to predict the presence of portal hypertension. LS measurement may allow prediction of the presence of large EV in patients with cirrhosis and may help to select patients for endoscopic screening^[8-11].

The purpose of this study was to determine if TE can be used to predict indirectly the presence of portal hypertension and the risk of variceal bleeding. At present the Baveno V and AASLD Consensus recommend screening all cirrhotic patients for EV. If LS measurement could predict the presence of large EV in patients with cirrhosis, we could select these patients for endoscopic screening.

MATERIALS AND METHODS

We studied 1000 consecutive patients with liver cirrhosis. Patients with hepatocellular carcinoma were excluded. In all patients, upper digestive endoscopy was performed by 10 experienced endoscopists (at least 300 upper digestive endoscopies performed). The time interval between endoscopy and TE evaluation was less than three months. EV were classified as: small (grade I) - small straight varices; medium (grade II) - enlarged tortuous varices occupying less than one third of the lumen; large (grade III) - large coil-shaped varices occupying more than one third of the lumen.

In accordance with EV diagnosed on endoscopy and the history of variceal bleeding we divided this batch into 2 groups: Group V0,1 - without EV and patients with grade 1 varices (647 cases); Group V2,3 - with significant portal hypertension (grade 2 and 3 EV) (353 cases).

We selected only those cases with EV and we divided them into 2 groups: Group no upper digestive bleeding (UDB) - without variceal hemorrhage (375 patients) and

Group UDB - (165 cases) with variceal bleeding.

In all patients we performed LS measurement by TE using a FibroScan device (Echosens, Paris). Measurements were performed in the right lobe of the liver through the intercostal spaces, on patients lying in the dorsal decubitus position with the right arm in maximal abduction. The tip of the transducer probe was covered with coupling gel and placed on the skin, between the rib bones at the level of the right lobe of the liver. The operator, assisted by an ultrasonic time-motion image, located a liver portion of at least 6 cm thick, free of large vascular structures. Once the measurement area had been located, the operator pressed the probe button to start an acquisition. Measurement depth was between 25 mm and 65 mm below the skin surface. Measurements which did not had a correct vibration shape or a correct follow up of the vibration propagation were automatically rejected by the software. Ten successful measurements were performed on each patient. The success rate (SR) was calculated as the ratio of the number of successful measurements over the total number of acquisitions. The results are expressed in kilopascal (kPa). The median value of the successful measurements was kept as representative of LS. Only LS measurements obtained with at least 10 successful measurements, with a SR of at least 60% and an IQR < 30% (IQR, the interquartile range interval, is the difference between the 75th and the 25th percentile, essentially the range of middle 50% of the data) were considered reliable.

Both the endoscopist and FibroScan operator were blinded to the result of TE evaluation and endoscopic evaluation of EV, respectively.

For a statistical study of quantitative variables, the mean and standard variation were calculated. T-tests were performed to compare mean values of LS in various subgroups. The diagnostic performance of LS measurements was assessed by using receiver operating characteristics (ROC) curves. ROC curves were thus built for the detection of various grades of EV and for predicting the risk of variceal bleeding. Optimal cut-off values for LS measurements were chosen to maximize the sum of sensitivity and specificity.

The statistical analysis was performed using Microsoft Excel (Microsoft Office 2007) and GraphPad Prism 5 programs.

RESULTS

The mean LS values in the 647 patients without or with grade 1 EV were statistically significantly lower than in the 353 patients with significant EV (26.29 ± 0.60 kPa *vs* 45.21 ± 1.07 kPa, $P < 0.0001$) (Figure 1).

Using the ROC curve we established a cut-off value of 31 kPa for the presence of significant EV, with 83% sensitivity (95% CI: 79.73%-85.93%) and 62% specificity (95% CI: 57.15%-66.81%), with 76.2% positive predictive value (PPV) (95% CI: 72.72%-79.43%) and 71.3% negative predictive value (NPV) (95% CI: 66.37%-76.05%)

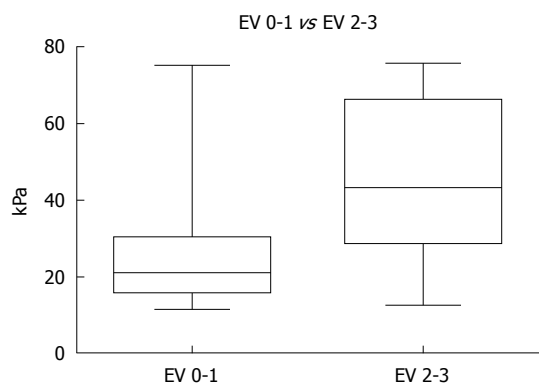


Figure 1 Mean liver stiffness values in patients with no or grade 1 esophageal varices as compared to those with significant esophageal varices (grade 2 and 3). Whiskers = minimum and maximum. EV: Esophageal varices.

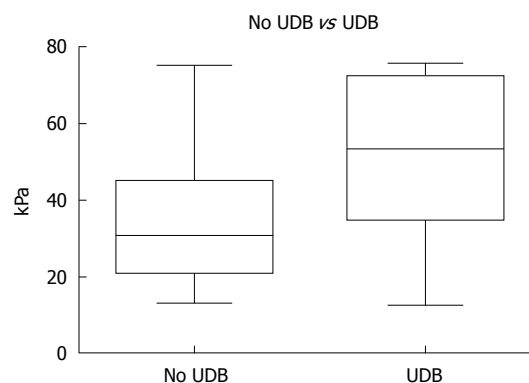


Figure 3 Mean liver stiffness values in patients with upper digestive bleeding due to variceal bleeding as compared to those without upper digestive bleeding. Whiskers = minimum and maximum. UDB: Upper digestive bleeding.

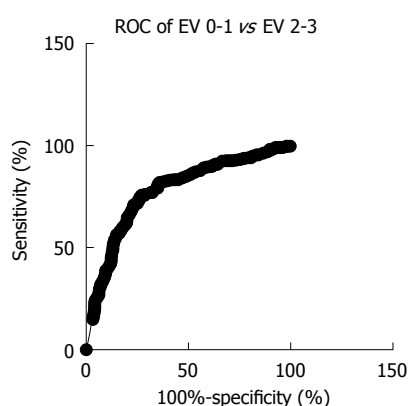


Figure 2 Predictive value of liver stiffness for the presence of at least grade 2 esophageal varices. ROC: Receiver operating characteristic.

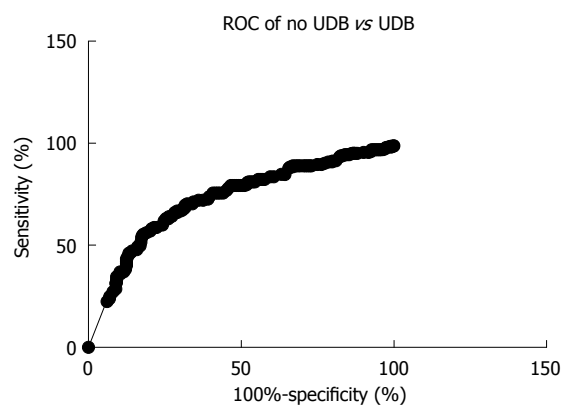


Figure 4 Predictive value of liver stiffness for upper digestive bleeding due to variceal bleeding. UDB: Upper digestive bleeding.

(AUROC 0.7807, $P < 0.0001$) (Figure 2).

The mean LS values in the group with a history of variceal bleeding (165 patients) were statistically significantly higher than in the group with no bleeding history (375 patients): 51.92 ± 1.56 kPa *vs* 35.20 ± 0.91 kPa, $P < 0.0001$ (Figure 3).

For a cut-off value of 50.7 kPa, LS had 53.33% sensitivity (95% CI: 45.42%-61.13%) and 82.67% specificity (95% CI: 78.45%-86.36%), with 82.71% PPV (95% CI: 78.5%-86.4%) and 53.66% NPV (95% CI: 45.72%-61.47%) (AUROC 0.7300, $P < 0.0001$) (Figure 4).

DISCUSSION

In previous studies, LS values < 19 kPa were highly predictive of the absence of significant EV ($\geq$ grade 2), the cut off values for the presence of grade 2 and 3 EV ranging from 27.5 to 35 kPa, and the cut off value for esophageal bleeding being 62.7 kPa^[12-14].

In other studies, LS measurement by TE was not accurate for the prediction of EV, with AUROC ranging from 0.76 to 0.84. Although sensitivity was good (71%-96%), specificity and PPV were low (60%-80% and 48%-54%, respectively) and overall accuracy was inferior as compared to simple tests like platelet count/spleen

diameter ratio^[11]. Another problem arising from these studies is the wide range of proposed cut offs, varying from 13.9 to 21.3 kPa for all varices, from 19 to 30 kPa for grade 2 varices and from 55 to 63 kPa for bleeding varices. The optimal cut offs therefore are still to be defined^[15,16].

Foucher *et al*^[13] (2006) assessed the accuracy of TE for the detection of large EV and the risk of variceal bleeding in patients with chronic liver disease. For the presence of EV grade 2 and 3, and esophageal bleeding, the cut offs were 27.5 and 62.7 kPa, respectively. The authors concluded that TE use for the follow-up and management of these patients could be of great interest and should be evaluated further.

Rudler *et al*^[17] reported their data on consecutive patients admitted to the intensive care unit with variceal hemorrhage who underwent hepatic venous pressure gradient (HVPG) measurement and TE. With an HVPG cut off > 20 mmHg (HVPG > 20 mmHg is an independent predictor of death in patients with cirrhosis and variceal bleeding), 8 patients were enrolled, but 4 could not undergo elastography because of severe ascites. Correlation between LS and HVPG was poor. The authors concluded that TE is unlikely to be of utility in this patient popula-

tion.

Klibansky *et al*^[18] was more successful in describing a useful application of TE to predict clinical outcomes in cirrhosis. Clinical endpoints were defined as the development of ascites or encephalopathy, variceal bleeding, development of hepatocellular carcinoma, or liver transplantation. Multivariate analysis indicated that the only independent predictors of outcome were Child-Pugh score and LS.

In 2009, Castéra *et al*^[19] showed that TE could be a valuable tool for the diagnosis of cirrhosis but cannot replace endoscopy for variceal screening.

Another study assessed the correlation between LS with FibroScan and HVPg in diagnosing significant portal hypertension in 150 patients who underwent a liver biopsy and hemodynamic measurements. In patients with significant portal hypertension (HVPg > 10 mmHg), AUROC for FibroScan was 0.945. The cut off value of 21 kPa accurately predicted significant portal hypertension in 92% of patients for whom measurements were successful^[20].

Due to the controversial results of all the studies described above we wanted to evaluate the value of TE for the prediction of EV and for the risk of bleeding in our patients.

The results of our study showed that TE is a useful technique for evaluating the presence of EV and hemorrhage prediction in cirrhotic patients. For a cut off value of 31 kPa, negative and PPVs were 76.2% and 71.3%, respectively. For > 31 kPa criterion, the cut off value was chosen to maximize the sum of sensitivity and specificity, whereas for > 40 kPa criterion we chose a cut off value to have a PPV of more than 85% with 77.8% sensitivity (95% CI: 74.57%-80.79%), 68.3% specificity (95% CI: 62.55%-73.68%), 86% PPV (95% CI: 83.18%-88.66%) and 55% NPV (95% CI: 49.60%-60.23%). For a cut-off value of 17.1 kPa, chosen to have a NPV close to 90%, we found the NPV to be 89.3%, with 43.2% PPV, 92.6% sensitivity and 33.5% specificity.

In clinical practice, such results could be of major relevance for the follow up of patients with cirrhosis. Cirrhosis places the patient at risk of clinical complications, such as portal hypertension, and variceal rupture is the second cause of death in cirrhosis, justifying early screening for EV. The usual means of diagnosing EV is upper gastrointestinal endoscopy. However, endoscopy can be considered invasive due to the technique and level of discomfort and the last recent consensus did not recommend endoscopic screening for the evaluation of bleeding risk in patients with grade 2 or 3 EV^[6,7]. Non-invasive methods for diagnosis need to be developed.

According to our data, in patients with TE values > 40 kPa at least 8/10 cases will have significant portal hypertension. Therefore it is reasonable to recommend prophylactic β -blocker therapy without endoscopy. On the other hand, in patients with TE values < 40 kPa, 5/10 cases will have significant EV (NPV 54.9%), thus we recommend endoscopic evaluation. Below the cut-off value of 17.1 kPa we do not recommend endoscopic evalua-

tion, since the chance of those patients having significant EV is only 1 in 10 (NPV 89.3%).

In our study we established an LS cut off value of 50.7 kPa for the risk of bleeding. A cut off value higher than 64 kPa correlates with a PPV > 90%, which means that those patients have a very high risk of bleeding despite primary prophylaxis with propranolol, with 77.75% sensitivity (95% CI: 73.64%-81.50%), 72.94% specificity (95% CI: 62.21%-82%), 93.88% PPV (95% CI: 90.96%-96.08%), 38.04% NPV (95% CI: 30.56%-45.96%). This data shows that 9 from 10 cases with TE values above 64 kPa will have variceal bleeding and we recommend prophylactic ligation in patients with LS values of more than 64 kPa.

In conclusion, LS measurement by means of TE is accurate for assessing the presence of large EV and the risk of variceal hemorrhage in cirrhotic patients.

COMMENTS

Background

The risk of variceal hemorrhage is related to the size of esophageal varices (EV). Therefore primary prevention of variceal bleeding applies in patients with previously diagnosed large EV (grade 2 or 3) detected by periodical upper digestive endoscopy. A generalized program of periodical upper tract endoscopy in these patients might result in a heavy economic burden even for developed countries. Furthermore, repeated examinations when not performed under profound sedation are often poorly accepted by patients who may refuse further follow up. Transient elastography (TE) may be used to predict the presence of portal hypertension. Liver stiffness (LS) measurement by means of TE may allow prediction of the presence of large EV in patients with cirrhosis and may help select patients for endoscopic screening.

Research frontiers

TE may be used to predict the presence of portal hypertension. LS measurement may allow prediction of the presence of large EV in patients with cirrhosis and may help to select patients for endoscopic screening. The purpose of this study was to determine if TE can be used to predict indirectly the presence of portal hypertension and the risk of variceal bleeding. Currently, the Baveno V and AASLD Consensus recommend screening all cirrhotic patients for EV. If LS measurement could predict the presence of large EV in patients with cirrhosis, we could select these patients for endoscopic screening.

Innovations and breakthroughs

The study showed that, in patients with TE values > 40 kPa at least 8/10 cases will have significant portal hypertension. Therefore it is reasonable to recommend prophylactic β -blocker therapy without endoscopy. In patients with TE values < 40 kPa, 5/10 cases will have significant EV [negative predictive value (NPV) 54.9%], thus endoscopic evaluation is mandatory. Below the cut-off value of 17.1 kPa endoscopic evaluation is not recommended, since the chance of those patients having significant EV is only 1 in 10 (NPV 89.3%). The research team established a LS cut off value of 50.7 kPa for the risk of bleeding. A cut off value higher than 64 kPa correlates with a positive predictive value > 90%, which means that those patients have a very high risk of bleeding despite primary prophylaxis with propranolol. The data shows that 9 from 10 cases with TE values above 64 kPa will have variceal bleeding and they recommend prophylactic ligation in patients with LS values of more than 64 kPa.

Applications

The authors recommend prophylactic β -blocker therapy without endoscopy in all patients with LS measurements > 40 kPa. In patients with LS measurements between 17.1 kPa and 40 kPa, considered to be "the grey zone", we recommend endoscopic evaluation of EV. Below the cut-off value of 17.1 kPa, endoscopic evaluation is not recommended since the chance of those patients to have significant EV is only 1 in 10 (NPV 89.3%). In patients with LS measurements higher than 64 kPa we recommend prophylactic band ligation of EV.

Terminology

TE is a new promising noninvasive and rapid method for the diagnosis and

quantification of liver fibrosis in patients with chronic liver disease. Some recent extensive studies have demonstrated that LS measurement by means of TE is a good alternative for liver biopsy. In cirrhotic patients, LS measurements range from 12.5 to 75.5 kPa.

Peer review

This is a nice study on the important topic of the non invasive diagnosis of large EV. Its strengths are in its size and what appears to be reliable data from TE.

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Increased intestinal permeability in inflammatory bowel diseases assessed by iohexol test

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Abstract

AIM: To study intestinal permeability (IP) and its relationship to the disease activity in patients with inflammatory bowel diseases (IBD) - Crohn's disease (CD) and ulcerative colitis (UC).

METHODS: Fifty-eight patients with active IBD (32 with CD and 26 with UC) and 25 healthy controls consented to participate in the study. The clinical activity of CD was estimated using the Crohn's Disease Activity Index (CDAI), and the endoscopic activity of UC using the Mayo scoring system. IP was assessed by the rise in levels of iohexol, which was administered orally (25 mL, 350 mg/mL) 2 h after breakfast. Three and six hours later serum (SIC mg/L) and urine (UIC g/mol) iohexol concentrations were determined by a validated HPLC-UV technique.

RESULTS: In the CD group, SIC values at 3 h (2.95 ± 2.11 mg/L) and at 6 h after ingestion (2.63 ± 2.18 mg/L) were significantly higher compared to those of healthy subjects (1.25 ± 1.40 mg/L and 1.11 ± 1.10 mg/L, respectively, $P < 0.05$). UIC (g/mol) values were also higher in patients, but the differences were significant only for UIC at 6 h. Significant positive correlation ($P < 0.05$) was found between the CDAI and IP, assessed by SIC at 3 h ($r = 0.60$) and 6 h ($r = 0.74$) after the ingestion. In comparison to controls, SIC and UIC of UC patients were higher in the two studied periods, but the differences were significant at 6 h only. Significantly higher values of SIC ($P < 0.05$) were found in patients with severe endoscopic activity of UC compared to those of patients with mild and moderate activity (3.68 ± 3.18 vs 0.92 ± 0.69 mg/L).

CONCLUSION: Serum levels of iohexol at 3 h and 6 h after its ingestion reflect increased IP, which is related to the disease activity in patients with IBD.

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Key words: Intestinal permeability; Iohexol test; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two

principal forms of inflammatory bowel disease (IBD), are characterized by a chronic inflammation of the gastrointestinal tract. Over the past decade there has been increasing recognition of the importance of both epithelial barrier function and innate immunity in the genesis of intestinal inflammation^[1-5].

Mucosal barrier function in IBD has been investigated in numerous studies and many of them, but not all^[6], have demonstrated increased intestinal permeability (IP) in some of the patients^[7-11]. Alterations of IP can be evaluated using several different probes such as disaccharides (lactulose, cellobiose), monosaccharides (mannitol), polyethyleneglycols of different molecular mass and ⁵¹Cr-ethylenediamine-tetraacetate (⁵¹Cr-EDTA). Studies have utilized different methods for the assessment of permeability, both in regard to administration procedures of the different probes and to outcome measures^[12,13].

So far, IP assessment in patients has been performed by measuring urine recovery of ingested permeability substrates. The urine test can be relatively uncomfortable and can lead to potential inaccuracy^[14,15] due to incomplete urine collection, renal dysfunction and variable hydration status of the tested subjects. In addition, some analytical difficulties in quantifying carbohydrates in urine have limited the widespread use of those permeability markers. Furthermore, urinary tract infections may compromise the recovery of sugar permeability markers^[16]. Measurement of the permeability substrates in plasma or serum could eventually reduce the problems with urine recovery and could potentially have a valuable role, particularly in pediatric patients.

The aim of the present study was to evaluate IP in patients with active IBD (CD and UC) by measuring serum and urine levels of water-soluble contrast medium iohexol, to assess the relationship of IP to the disease activity, and to compare the reliability of serum vs urine levels of iohexol as an IP disease marker.

MATERIALS AND METHODS

Patients and controls

The study included 58 patients with active IBD: 32 patients with CD (16 males, 16 females, mean age 38.9 years: range 18-70 years) and 26 patients with UC (11 males, 15 females, mean age 41.5 years: range 21-70 years), hospitalized in the Clinical Centre of Gastroenterology, University Hospital Queen Joanna, Sofia. Diagnosis was based on the commonly accepted clinical, endoscopic and histological criteria. Disease activity of CD patients was estimated using the Crohn's Disease Activity Index (CDAI); values greater than 150 were accepted as a marker for clinical activity^[17]. The endoscopic activity (EA) of UC was assessed using the Mayo scoring system (findings on endoscopy): 0: normal or inactive disease; 1: mild disease (erythema, decreased vascular pattern, mild friability, erosions); 2: moderate disease (marked erythema, lack of vascular pattern, friability); 3: severe disease (spontaneous bleeding, ulceration) (Table 1). CD patients were divided in two subgroups with regard to the endoscopic activity score: "EA

Table 1 Characteristics of patients with Crohn's disease and ulcerative colitis

Variables	CD (n = 32)	UC (n = 26)
Males/females	16/16	11/15
Age (yr), mean (range)	38.9 (18-70)	41.5 (21-70)
Duration of the disease (yr), mean (range)	6 (1-31)	6 (1-26)
Crohn's disease		
Area of involvement of the GI tract		
Small intestine only (L1)	4	
Large intestine only (L2)	7	
Ileo-colonic (L3)	21	
UGIT involvement (L4)	0	
Clinical activity		
CDAI, median (range)	211 (156-344)	
CDAI 151-220	17	
CDAI 221-400	15	
CDAI > 400	0	
Ulcerative colitis		
Extent (E)		
Proctitis (E 1)		2
Left-side (distal) colitis (E 2)		12
Total colitis (E 3)		12
Endoscopic activity-Mayo scoring system (EA)		
Endoscopic remission (EA 0)		0
Mild (EA 1)		6
Moderate (EA 2)		5
Severe (EA 3)		15

GI: Gastrointestinal; UGIT: Upper gastrointestinal tract; CDAI: Crohn's Disease Activity Index.

3" - severe and "EA 1+2" - mild and moderate endoscopic activity.

The location and behavior of CD, and extent and severity of UC, were classified using the modified Montreal classification^[18]. According to CDAI, all CD patients had active disease (CDAI > 150). All patients with UC had endoscopic features of an active disease. Twenty-five healthy persons (18 males, 7 females, mean age 40.6 years: range 25-68 years) were recruited as a control group. None of them had signs or symptoms of gastrointestinal disorders or renal diseases. None of the investigated subjects took alcohol, non-steroidal anti-inflammatory drugs or any other medications (antidepressants, anticholinergics, metoclopramide, lactulose) that have the potential to affect gastrointestinal motility, for at least 2 wk before the test. Renal function, assessed by serum creatinine levels and calculated Glomerular Filtration Rate, was normal in all investigated subjects (Table 1).

The study was approved by the Ethics Committee of University Hospital Queen Joanna. Written informed consent was obtained from all patients and control subjects.

Assessment of the IP

Iohexol (Omnipaque™, Nycomed-General Electric) was administered orally (25 mL of 350 mg/mL injection solution) in the morning, 2 h after breakfast, immediately after voiding. Drinking was not allowed for the next 3 h. Food was permitted after 5 h. Blood and urine samples

were collected at 3 and 6 h after the iohexol ingestion. Serum was separated within 45 min after the blood withdrawal by centrifugation at 1 500 *g* for 10 min. Serum and urine were kept frozen at -20°C until analysis. Iohexol concentrations were determined by a validated high pressure liquid chromatography technique. Briefly, sample preparation consisted of protein precipitation; separation was performed on a C8 column with a mobile phase of 5% aqueous acetonitrile and detection at 240 nm. Selectivity was confirmed by comparing the signal in blank and spiked samples in 12 individual sources of human serum and urine. Accuracy and precision (within-run and between-runs) were within 12%; extraction recovery was over 90%; linearity range was 0.25-10.00 mg/L for serum samples and 2.50-700.00 mg/L for urine samples; $R^2 > 0.998$. Stability was also validated accordingly. Urine results were presented as iohexol/creatinine ratios (g/mol). Results of the control group (mean + 2SD) were used as a cut-off level for increased IP at 95% confidence interval, and values exceeding that cut-off were considered abnormal.

Statistical analysis

Statistical methods used: descriptive statistics, nonparametric ANOVA (Kruskal-Wallis test), nonparametric Mann-Whitney *U* test for overall testing the difference between subject groups, nonparametric Wilcoxon test for the difference between pairs of groups, Pearson's test (*r*) for correlation. Each hypothesis was tested at a level of significance of 0.05.

RESULTS

All permeability tests were well tolerated and no side effects were reported. Based on the results obtained from healthy subjects, values of SIC over 4.0 and 3.3 mg/L at 3 and 6 h respectively, as well as values of UIC over 35.5 and 31.4 g/mol at 3 and 6 h after ingestion, were accepted as abnormal (cut-offs). One control subject had slightly increased IP at 3 h, and two control subjects at 6 h, post-iohexol ingestion.

The results from applied Kruskal-Wallis test showed that the factor "group" is significant ($P < 0.05$). Mann-Whitney *U* test results indicated that in the group of CD patients the mean values of SIC at 3 h (2.95 ± 2.11 mg/L) and at 6 h (2.63 ± 2.18 mg/L) post-ingestion were significantly higher than those in the control group (1.25 ± 1.40 mg/L and 1.11 ± 1.10 mg/L, respectively). Abnormal IP was found in 10 patients at 3 h (31%) and in 16 patients at 6 h (50%) after iohexol intake. Urine recovery of iohexol was also higher in patients 3 h (18.09 ± 13.13 g/mol) and 6 h (36.92 ± 27.68 g/mol) post-ingestion compared to the control group (14.64 ± 10.44 g/mol and 14.18 ± 7.78 g/mol, respectively), but the difference was significant only for the mean UIC values at 6 h.

In the UC group, the mean serum levels (1.57 ± 1.55 mg/L and 2.49 ± 2.80 mg/L) and urine recovery of

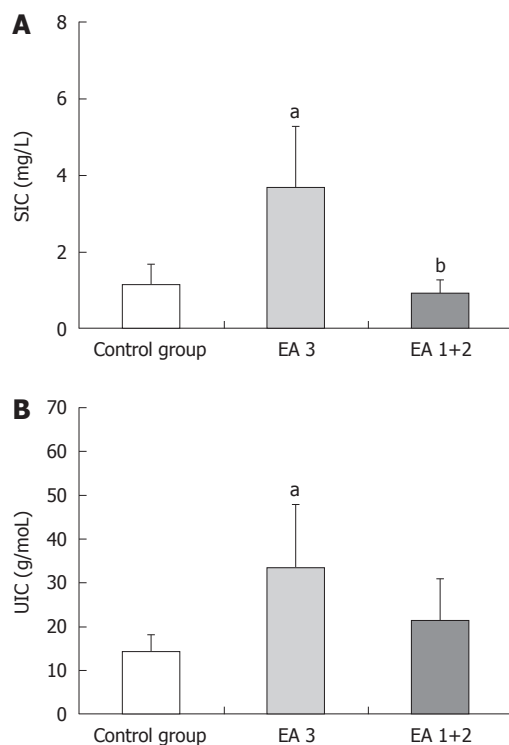


Figure 1 Serum iohexol concentrations (A) and urine iohexol concentrations (B) (mean ± SE) at 6 h post-ingestion according to Endoscopic Activity Mayo score (EA) of patients with ulcerative colitis. EA3: Severe endoscopic activity; EA 1+2: Mild and moderate endoscopic activity. ^a $P < 0.05$, SIC and UIC vs control group; ^b $P < 0.05$, EA 3 vs EA 1+2 group. SIC: Serum iohexol concentrations; UIC: Urine iohexol concentrations.

iohexol (9.86 ± 9.26 g/mol and 27.76 ± 25.18 g/mol) at 3 and 6 h after its ingestion, respectively, were higher than those for healthy controls, but the differences were significant only at 6 h for both parameters. IP was established as abnormal in 3 (11%) and 8 (31%) patients with UC at 3 and 6 h post-iohexol ingestion, respectively.

There was a significant positive correlation between the parameters SIC and CDAI in the group of CD patients at 3 h ($r = 0.60$) and at 6 h ($r = 0.74$) post-ingestion, while the correlation between CDAI and UIC was insignificant both at 3 h ($r = 0.48$) and at 6 h ($r = 0.32$) post-ingestion of iohexol.

In the subgroup "EA 3" of patients with severe endoscopic activity of UC ($n=15$), the mean values of SIC at 6 h were significantly higher (3.68 ± 3.18 mg/L), compared to those of the control group (1.11 ± 1.10 mg/L), while the values (0.92 ± 0.69 mg/L) for the patient subgroup "EA 1+2" ($n = 11$) with mild and moderate endoscopic activity did not differ from the controls (Figure 1A). The mean UIC values for patient subgroup "EA 3" at 6 h were also significantly higher (33.60 ± 28.42 g/mol) compared to those of the control group (14.64 ± 10.44 g/mol), and mean UIC values of the subgroup "EA 1+2" (19.78 ± 18.25 g/mol) were slightly but insignificantly higher from the control values (Figure 1B).

The permeability disturbances were five-fold more frequent in the subgroup of patients with severe activity (47%) in comparison to the subgroup of patients with

mild and moderate activity (9%).

DISCUSSION

Altered barrier function in IBD is documented in experimental and clinical studies, but it is difficult to compare the results of IP due to the existing variability in the chemical nature of the chosen candidate markers in the experimental design and methodology. It is considered that the larger molecular weight markers reflect the changes predominantly in paracellular permeability, while smaller ones register transcellular transfer changes. Paracellular permeability across the epithelial cell monolayers is regulated primarily by the tight junctions (TJs) that encircle the apical poles of the epithelial cells^[1,7]. The epithelium in the inflamed intestinal segments of patients with CD is characterized by a reduction of the TJ strands, strand breaks, and alterations of the TJ proteins. In patients with UC the epithelial leaks appear early due to micro-erosions resulting from upregulated epithelial apoptosis and in addition to a prominent increase of claudin-2^[3]. Immune regulation of the epithelial functions by cytokines may cause a barrier dysfunction not only by the TJ impairments but also by apoptotic leaks, transcytotic mechanisms and mucosal gross lesions^[4].

In our study, IP in patients with IBD was examined by measuring of the serum levels and urine recovery of iohexol, following oral administration. Andersen *et al.*^[19] demonstrated that water-soluble radiographic contrast media could be of use for evaluation of the altered intestinal barrier function. The contrast agent iohexol is a moderately large molecule (molecular weight 821 Dalton) with a low absorption under normal conditions, and enhanced absorption through the inflamed intestinal mucosa. It does not bind to serum proteins and is filtered through the glomerulus without indications of tubular secretion or reabsorption. Until now, to the best of our knowledge, serum levels of iohexol have not been used for the assessment of IP. The results of this study demonstrate that iohexol permeation through the intestinal mucosa is significantly increased in both CD and UC patients. Using different probes, similar findings have also been reported in numerous studies both in adult and pediatric populations with IBD^[7-11,20-24]. The findings of this study support the understanding that patients with active IBD (including both CD and UC) have a mucosal barrier dysfunction, which can be assessed by measurement of IP. The permeability alterations were more frequent in CD than in UC patients: increased iohexol absorption as a marker for the abnormal IP was established in 50% of CD and in 31% of UC patients, figures which were significantly more frequent in comparison to the healthy subjects. Taking into account the fact that 3 h after ingestion iohexol is located in the large intestine^[7], the higher SIC and UIC at 6 h after ingestion in patients with UC in our research suggest that this test can be used for evaluation of the colon permeability also.

The relationship between increased IP and disease ac-

tivity in IBD has been established in some studies^[9,11,20,21]. IP in CD patients is increased proportionally to the disease activity; it can be used to predict the clinical relapse of the disease (due to subclinical mucosal inflammation) and to assess prognosis^[25,26]. However, the data in the literature are contradictory, most probably due to the usage of different permeability probes as mentioned above. Our study demonstrates that the permeation of iohexol through the intestinal mucosa, evaluated by serum concentrations, correlates positively with the disease activity in CD patients. These results are in agreement with data reported by Halme *et al.*^[7,20] for increased IP of iohexol (measured by its urinary recovery) and its correlation with the clinical disease activity indices. Furthermore, Halme *et al.*^[27] concluded that the iohexol test is a superior activity marker compared to the lactulose-mannitol test in patients with IBD. We established a relationship between serum levels of iohexol and the endoscopic activity score for UC patients. Using different permeability markers, Miki *et al.*^[11], Arslan *et al.*^[23] and Casellas *et al.*^[24] found a relationship between IP and disease activity in IBD patients. However, other investigators did not establish such a correlation^[8,28,29]. Our data for significant positive correlation between iohexol penetration through the intestinal mucosa and disease activity support the hypothesis of the important role of the impaired intestinal barrier in the pathogenesis of active IBD^[2,3,5].

In conclusion, the water-soluble contrast medium iohexol is a suitable marker for assessing the gut barrier function as its penetration through the intestinal mucosa is increased in patients with active IBD (both CD and UC) and is related to the disease activity. In our study, serum iohexol concentration appears to be a superior marker of altered IP, compared to urinary iohexol level. Measurement of a single serum sample of iohexol 6 h following its oral administration makes the proposed permeability test more convenient and provides a possibility for the assessment of altered barrier function in both small and large intestine.

COMMENTS

Background

Increased intestinal permeability (IP) has been implicated in the pathogenesis of the inflammatory bowel diseases (IBD). Mucosal barrier function has been investigated by the measuring of urine recovery of ingested permeability substrates, which is relatively uncomfortable and could lead to potential inaccuracy. To clarify the role of barrier dysfunction and to introduce better diagnostic procedure, the authors decided to evaluate IP in patients with active IBD by measuring serum and urine levels of water-soluble contrast medium iohexol, to assess the relationship of IP to disease activity, and to compare the reliability of serum vs urine levels of iohexol as an IP disease marker.

Research frontiers

In the present study the relationship between IP and disease activity in patients with active IBD was investigated by using the iohexol test. The findings support the hypothesis that, by affecting the penetration of macromolecules and pathogens, the permeability disorders are linked to intestinal inflammation and play a role in mucosal damage in IBD.

Innovations and breakthroughs

Mucosal barrier function in IBD has been investigated in numerous studies which differ with respect to methods for the permeability assessment, both in

regard to administration procedures of the different probes and to outcome measures. Measurement of permeability substrates in plasma or serum may reduce the problems with urine recovery and might potentially have a valuable role.

Applications

The assessment of permeability alterations with the suitable, easy to perform, and convenient iohecol test provides a platform for therapeutic modulation of the gut barrier function for better control of IBD patients.

Terminology

IP reflects the integrity of the intestinal mucosa to prevent penetration of macromolecules and bacterial antigens from the gut lumen. Tight junctions are continuous, circumferential, belt-like structures that encircle the apical poles of the epithelial cells. They are a key regulator of paracellular permeability across the intestinal epithelium. Iohecol is a non-ionic, water-soluble contrast agent.

Peer review

The authors found that patients with active ulcerative colitis and Crohn's disease had increased serum and urinary concentration of iohecol, indicating disruptive IP. There was a correlation between severity of intestinal leakiness and disease activity. The authors concluded urinary and serum iohecol are acceptable methods of assessing IP. The authors concluded that IP disrupted in patients with active IBD. It is an interesting study and data appear to support the conclusion.

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Pre-operative factors that can predict neoplastic polypoid lesions of the gallbladder

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Abstract

AIM: To investigate the preoperative factors that can predict neoplastic polypoid lesions of the gallbladder (PLGs) as well as malignant PLGs.

METHODS: A retrospective analysis was conducted on the 210 consecutively enrolled patients who underwent cholecystectomy due to a PLG larger than 10 mm, as was determined by preoperative trans-abdominal ultrasonography or endoscopic ultrasonography. We ana-

lyzed the medical, laboratory, radiologic data and the pathologic results.

RESULTS: In 210 cases, 146 had non-neoplastic polyps (69.5%) and 64 cases were neoplastic polyps (30.5%). An older age (≥ 65 years), the presence of diabetes mellitus (DM) and the size of polyp (≥ 15 mm) were revealed to be independent predictive variables for neoplastic polyps with odd ratios (OR) of 2.27 ($P = 0.044$), 2.64 ($P = 0.021$) and 4.94 ($P < 0.01$), respectively. Among the neoplastic PLGs, an older age (≥ 65 years), the presence of DM and polyp size (≥ 15 mm) were associated with malignancy with ORs of 4.97 ($P = 0.005$), 6.13 ($P = 0.001$) and 20.55 ($P < 0.001$), respectively.

CONCLUSION: Among patients with PLGs larger than 10 mm in size, higher risk groups such as elderly patients more than 65 years old, those with DM or a large polyp size (≥ 15 mm) should be managed by cholecystectomy.

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Key words: Gallbladder; Polyp; Neoplastic; Cholecystectomy; Diabetes; Pre-operative factors

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Cha BH, Hwang JH, Lee SH, Kim JE, Cho JY, Kim H, Kim SY. Pre-operative factors that can predict neoplastic polypoid lesions of the gallbladder. *World J Gastroenterol* 2011; 17(17): 2216-2222 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i17/2216.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i17.2216>

INTRODUCTION

A polypoid lesions of the gallbladder (PLGs) is defined

as any elevated lesion of the mucosal surface of the gallbladder wall. Sonographers have described PLGs as an image with similar echogenicity as that of the gallbladder wall; the lesion projects into the lumen and it is fixed, lacks displacement, it may or may not have a pedicle and it shows no acoustic shadow on ultrasonography^[1-3]. The prevalence of PLGs varies from 0.3% to 12% in healthy adults who undergo abdominal ultrasonography (US)^[4-11]. Although the exact prevalence of PLGs is not clear, the detection of PLGs has been increasing according to the more frequent use of abdominal imaging. Most of the PLGs that are without symptoms are non-neoplastic lesions, but a small portion of them are found to be malignant or premalignant neoplasms. The incidence of malignant polyps has varied from 1% to 20% of the resected PLGs among diverse study populations in previous reports^[2,12-17]. The largest PLG series was a review of 172 surgically resected cases, and this showed that the most common type of PLG was the cholesterol polyp (62.8%). They also reported that 7% were inflammatory polyps, 7% were hyperplasia, 5.9% were adenoma, 9.6% were miscellaneous and 7.7% were malignant polyps in the study population^[18]. Due to the considerable incidence of malignant polyps among the PLGs, surgical resection, including laparoscopic cholecystectomy, is widely accepted as the treatment of choice for PLGs that are more than 10 mm in size^[18]. This surgical treatment guideline has been supported by many previous published reports^[14,15,19]. However, the number of non-neoplastic polyps that are unnecessarily resected exceeds more than 3 times the number of neoplastic polyps when the resected polyps are in accordance with the above mentioned guideline^[20]. For this reason, some clinicians hesitate to recommend an operation based on this guideline.

Over the last 10 years, several interesting small trials have attempted to determine the endoscopic or transabdominal ultrasonographic features of neoplastic gallbladder polyps, as compared with those of nonneoplastic polyps^[10,11,21-23]. However, these sonographic findings have several limitations such as a mixed component of a benign nature, the lack of standardization and interobserver discrepancy^[20].

With this background, this study aimed to reveal the clinical and sonographic predictive findings of neoplastic PLGs, including malignant PLGs, in patients who have PLGs larger than 10 mm. We also tried to demonstrate the guidelines for the decision making for the surgical management of incidentally diagnosed gallbladder polyps.

MATERIALS AND METHODS

Patients

We performed a retrospective analysis of the consecutively enrolled patients who were diagnosed with a PLG larger than 10 mm by preoperative trans-abdominal ultrasonography or endoscopic ultrasonography (EUS) between March 1, 2003 and April 30, 2009 at Seoul National University Bundang Hospital. The study protocol

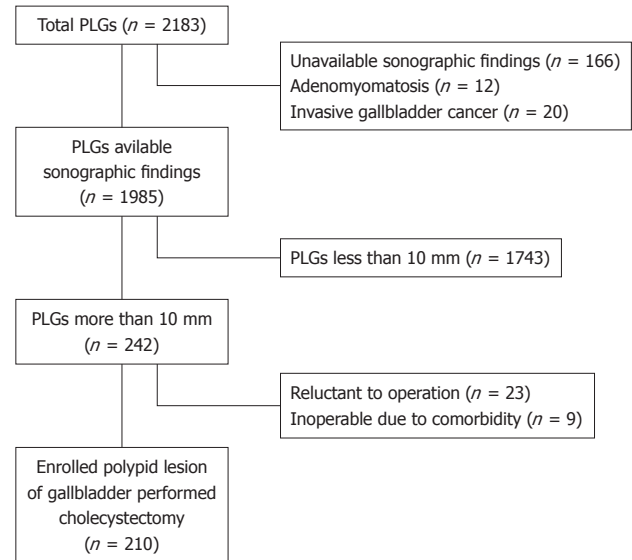


Figure 1 A diagram of the patients' enrollment. PLGs: Polypoid lesions of the gallbladder.

was approved by the Institutional Review Board of our hospital. According to our institution's policy, we recommended cholecystectomy to all patients who had a PLG that was more than 10 mm in size if they were in an operable condition. During the study period, a total of 2281 cases of PLG were diagnosed. Among them, 12 definite adenomyomatosis lesions with a sonographic "comet tail sign" and 20 lesions that were suspected of being gallbladder cancer that had invaded the liver or other adjacent organs were excluded. The 166 cases that did not have sonographic findings available or where polyps were measured by different sonographic equipment were excluded. Among the remaining cases, 1743 patients with small polyps (smaller than 10 mm) and 31 patients who did not undergo an operation were also excluded. Therefore, 210 patients who underwent cholecystectomy were ultimately analyzed in this study (Figure 1).

Based on the final diagnosis of the pathologic reports, all the polyps were divided into 2 groups: the non-neoplastic polyps (chronic cholecystitis, inflammatory polyps, adenomyomatosis, cholesterolosis or cholesterol polyps) and the neoplastic polyps (adenomatous polyps with low grade dysplasia, adenomatous polyps with high grade dysplasia, adenocarcinoma)^[24].

The following parameters of all patients were recorded and analyzed: the demographic features, including age, gender, a smoking history, a history of drinking alcohol, the presence of diabetes mellitus (DM), the presence of hypertension, clinical symptoms, measurements of obesity, a complete blood count, a routine chemistry panel, the fasting glucose level and the lipid profiles. The body mass index (BMI) was calculated by dividing the weight in kilograms by the square of the height in meters. Obesity was defined as a BMI > 25 kg/m² according to the Asian-Pacific criteria for obesity^[25]. Clinical symptoms were defined as abdominal pain that was compatible with biliary colic, such as right upper quadrant pain with or

without radiation pain that becomes aggravated with eating a fatty meal.

The radiologic reports were retrospectively reviewed by one experienced radiologist to describe and record the polyp size, the echogenicity, the echo pattern, the number of lesions, the location of lesion, lesion combined with gallbladder stones, the size change of the lesion and the duration of the size change. The histologic findings of all the resected specimens were retrospectively reviewed by one experienced pathologist.

Equipment and the definition of the sonographic findings

Abdominal sonography was performed by well trained sonographers who used 6-2 MHz curvilinear transducers with IU 22 or HDI 5000 units (Phillips). An EUS (endoscopic ultrasonogram) was obtained with 7.5-MHz or 12-MHz radial sector scan transducers (EUS-2000, Olympus Optical Co.), and these procedures were performed by 2 well-trained endosonographers. The EUS probe was advanced to the second portion or bulb of the duodenum and the gallbladder was scanned *via* the water-filled balloon method. All the sonographic findings of the patients were reviewed by two experienced radiologists.

The size of the polypoid lesion was measured by assessing the long diameter of the largest polypoid lesion. The echogenicity was determined on the ultrasonogram by comparing it with the echogenicity of the adjacent liver. For some cases that had a severe fatty liver, the echogenicity of the lesion was compared with the echogenicity of the kidney in same ultrasonographic series of the case. We classified the echogenicity into 3 categories: “hypoechoic”, “isoechoic” and “hyperechoic”. The surface pattern of the polypoid lesions was divided into 2 groups: “smooth” and “nodular”^[26]. The internal echo pattern of the polypoid lesions was divided into 2 categories: “homogeneous” and “inhomogeneous”. The number of polyps was divided into 2 categories: “multiple” and “solitary”. The patients with multiple polyps that consisted of both neoplastic and non-neoplastic polyps in one specimen were classified as having neoplastic polyps. The shape of the polypoid lesions was classified to 2 categories: “pedunculated” and “sessile”. Hyperechoic spots were defined “a single 1-5 mm, highly echogenic dot”, or “partial aggregates of 1-3 mm sized, multiple, highly echogenic spots”^[26].

Statistical analysis

Continuous variables are presented as the mean $\pm$ SD, and categorical variables are summarized as frequencies and percents. The variables were compared assuming a 95% probability for rejection of the null hypotheses. Fisher's exact test, Pearson's χ^2 test and student's *t*-test were used, when appropriate, to calculate the statistical significance of the different demographic and clinical variables. Multivariate binary logistic regression analysis was performed to determine the significance of the various predictive variables that were found to be significant by univariate

analysis. *P* values of < 0.05 were deemed as significant. All the statistical analyses were carried out using SPSS 15.0 software (SPSS, Chicago, Illinois, USA).

RESULTS

Clinical and sonographic characteristics of the patients

Of the 210 patients, 145 had non-neoplastic polyps (69.0%) and 65 had neoplastic polyps (31.0%). The histological diagnosis of the resected PLGs revealed that 54 cases (25.7%) were chronic cholecystitis, 3 cases (1.4%) were inflammatory polyps, 78 cases (37.1%) were cholesterol polyps, 10 cases (4.8%) were adenomatous, 29 cases (13.8%) were adenoma with low grade dysplasia, 6 cases (2.9%) were adenoma with high grade dysplasia and 30 cases (14.3%) were adenocarcinoma.

We compared the clinical and laboratory features between the non-neoplastic polyps group and the neoplastic polyps group. The results are described in Table 1. The mean age, the proportion of DM patients and the mean serum alanine transferase (ALT) level were higher in the neoplastic polyp group than that in the non-neoplastic group ($P < 0.001$, $P < 0.001$, $P = 0.041$, respectively). Yet no significant difference was found for gender, medical history and the other laboratory findings between the two groups.

For the sonographic findings, the mean sonographic diameters of the polyps were 13.5 ± 4.5 mm and 22.1 ± 11.1 mm for the non-neoplastic group and the neoplastic group, respectively ($P < 0.001$). In addition, the inhomogeneous echo pattern ($P = 0.019$), a solitary lesion ($P = 0.002$) and a nodular surface pattern of the polyps ($P < 0.001$) revealed significant correlation with neoplastic polyps (Table 1).

For the detailed analysis, maximum diameter was divided to 2 categories by use of receiver-operator characteristic (ROC) curves. At a cutoff value of 15 mm diameter of PLGs' size, the area under the ROC curve (AUC) had the highest sensitivity and specificity. (70.8%, 75.9%, Figure 2).

Predictive variables for neoplastic PLGs

On the univariate analysis, we obtained several important predictive clinical and sonographic values such as an age > 65 years, the presence of DM, the ALT level, a larger sonographic size (≥ 15 mm), solitary lesions and a nodular sonographic surface pattern (Table 1). On multivariate analysis, an older age (≥ 65 years), the presence of DM and polyp size (≥ 15 mm) were found to be the independent predictive variables for neoplastic polyps [odds ratios (OR) = 2.27, $P = 0.044$, OR = 2.64, $P = 0.021$ and OR = 4.94, $P < 0.001$, respectively]. A nodular surface pattern was found to have an association with neoplastic polyps, with borderline significance (OR = 2.31, $P = 0.058$) (Table 2).

Predictive variables for malignant PLGs

In addition, we subdivided the neoplastic group into two

Table 1 Comparative data for the prevalence of the demographic, laboratory and sonographic findings between the non-neoplastic polyp group and the neoplastic polyp group (mean $\pm$ SD) *n* (%)

	Total (<i>n</i> = 210)	Non- neoplastic (<i>n</i> = 146)	Neoplastic (<i>n</i> = 64)	<i>P</i>
Age (yr)	51.8 $\pm$ 13.7	49.1 $\pm$ 12.3	57.9 $\pm$ 14.7	< 0.001
Age > 65 yr	49 (23.3)	22 (15.1)	27 (42.2)	< 0.001
Gender, male	109 (51.9)	77 (52.7)	32 (50.0)	0.785
BMI (kg/m ²)	24.0 $\pm$ 2.97	23.9 $\pm$ 3.01	24.1 $\pm$ 2.89	0.620
Obesity	79 (38.2)	53 (36.6)	26 (41.9)	0.465
Hypertension	34 (16.3)	20 (13.7)	14 (22.2)	0.126
Diabetes mellitus	46 (21.9)	21 (13.0)	27 (42.1)	< 0.001
Hypercholesterolemia	77 (36.7)	57 (39.0)	20 (31.3)	0.135
RUQ pain	37 (17.6)	24 (16.4)	13 (20.3)	0.498
Total bilirubin (g/dL)	1.22 $\pm$ 3.41	0.91 $\pm$ 0.41	1.93 $\pm$ 6.17	0.189
ALP (g/dL)	69.4 $\pm$ 60.9	62.5 $\pm$ 20.2	84.7 $\pm$ 104.7	0.097
AST (IU/dL)	33.2 $\pm$ 61.9	26.2 $\pm$ 21.4	49.2 $\pm$ 105.9	0.090
ALT (IU/dL)	34.5 $\pm$ 42.0	29.1 $\pm$ 23.3	47.0 $\pm$ 66.2	0.041
Size (mm)	16.1 $\pm$ 8.20	13.5 $\pm$ 4.5	22.1 $\pm$ 11.1	< 0.001
Size > 15 mm	78 (37.1)	33 (22.3)	45 (70.3)	< 0.001
Location				0.977
Fundus	156 (74.3)	109 (74.7)	47 (73.4)	
Body	44 (21.0)	30 (20.5)	14 (21.9)	
Neck	10 (4.8)	7 (4.8)	3 (4.7)	
No. of polyps				0.002
Multiple	76 (36.2)	63 (43.2)	13 (20.3)	
Solitary	134 (63.8)	83 (56.8)	51 (79.7)	
Hyperchoic spots				0.315
No	172 (81.9)	117 (80.1)	55 (85.9)	
Yes	38 (18.1)	29 (19.9)	9 (14.1)	
Echogenicity				0.125
Anechoic or hyperechoic	130 (61.9)	96 (65.8)	34 (53.1)	
Hypoechoic or isoechoic	80 (38.1)	50 (34.2)	30 (46.9)	
Echo pattern				0.093
Homogeneous	115 (52.9)	85 (58.2)	30 (46.9)	
Inhomogeneous	95 (45.2)	60 (41.1)	35 (54.7)	
Sonographic surface pattern				< 0.001
Smooth surface	174 (82.9)	131 (89.7)	43 (67.2)	
Nodular surface	36 (17.1)	15 (10.3)	21 (32.8)	

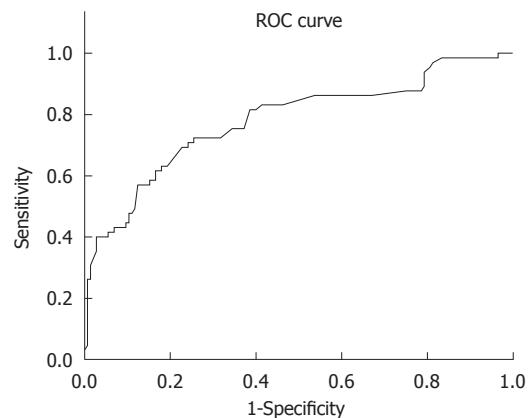
BMI: Body mass index; RUQ: Right upper quadrant; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase. Obesity: BMI higher than 25 kg/m².

groups according to their histologic results. The polyps that contained adenocarcinoma were classified as the malignant PLGs group and the other neoplastic polyps were classified as the benign PLGs group. We also compared the clinical and sonographic variables to discriminate the malignant PLGs group from the benign group. On univariate analysis, the important predictive clinical and sonographic values for malignant polyps were an older age (≥ 65 years, $P = 0.02$), the presence of DM ($P < 0.001$), the ALT level ($P = 0.033$), a larger sonographic size (≥ 15 mm, $P < 0.001$) and an inhomogeneous echo pattern ($P = 0.016$) (Table 3). But on multivariate analysis, it was observed that an older age (≥ 65 years), the presence of DM and polyp size (≥ 15 mm) had statistical significance

Table 2 Results of the multivariate logistic regression analysis for the factors that were significantly associated with neoplastic polypoid lesions of the gallbladder on univariate analysis

	Hazard ratio	95% CI	<i>P</i> -value
Age ≥ 65 yr old	2.27	1.02-5.06	0.044
Gender, male	1.08	0.57-2.51	0.617
DM	2.64	1.15-6.03	0.021
ALT level	1.008	0.99-1.02	0.168
Polyp size > 15 mm	4.94	2.43-10.02	< 0.001
Solitary polyp	0.59	0.26-1.33	0.205
Nodular surface pattern	2.31	0.97-5.50	0.058

DM: Diabetes mellitus; ALT: Alanine transaminase.

**Figure 2** Receiver-operator characteristic curve of the sonographic size of the polypoid lesions of the gallbladder.

with the malignant PLGs group (OR = 4.97, $P = 0.005$, OR = 6.13, $P = 0.001$, OR = 20.55, $P < 0.001$, respectively) (Table 4).

For a more detailed analysis of the chronological change of the neoplastic polyps, we classified all the cases into three subgroups: the adenoma with low grade dysplasia group; the adenoma with high grade dysplasia group; and the adenocarcinoma group. After this subgroup analysis, we found a linear stepwise increase in the mean age of each group; adenoma low grade dysplasia, high grade dysplasia and adenocarcinoma. The difference of the mean age was 18.9 years between the adenoma with low grade dysplasia group (46.4 ± 13.4 years) and the adenocarcinoma group (65.3 ± 18.0 years) ($P < 0.001$), and the difference of the mean age was 13.2 years between the high grade dysplasia group (52.1 ± 7.4 years) and the adenocarcinoma group ($P = 0.004$) (Figure 3).

DISCUSSION

GB polyps larger than 10 mm in size have generally been recommended for surgical resection despite of the large portion of non-neoplastic polyps among them. Because the current data for making the preoperative differentiation between neoplastic and non-neoplastic polyps is limited, a practical guideline was lacking to decide when to perform cholecystectomy. In this study, we tried to

Table 3 Comparative data for the prevalence of the demographic, laboratory and sonographic findings between the benign polyp group and the malignant polyp group for the 65 neoplastic polypoid lesions of the gallbladder (mean $\pm$ SD) *n* (%)

	Total (<i>n</i> = 65)	Benign (<i>n</i> = 35)	Malignant (<i>n</i> = 30)	<i>P</i>
Age (yr)	49.8 $\pm$ 13.5	47.2 $\pm$ 12.4	65.6 $\pm$ 7.39	< 0.001
Age > 65 yr	51 (24.3)	31 (17.2)	20 (66.7)	0.002
Gender, male	109 (51.9)	95 (52.6)	14 (46.7)	0.535
BMI (kg/m ²)	23.9 $\pm$ 2.97	24.0 $\pm$ 3.03	23.8 $\pm$ 2.66	0.835
Obesity	79 (38.2)	67 (37.2)	12 (40.0)	0.583
Hypertension	34 (16.3)	25 (13.8)	9 (30.0)	0.244
Diabetes mellitus	46 (21.9)	25 (13.8)	21 (70.0)	< 0.001
Hypercholesterolemia	19 (29.2)	10 (28.6)	7 (10.9)	0.830
RUQ pain	13 (6.2)	6 (4.1)	7 (10.9)	0.534
Total bilirubin (g/dL)	1.22 $\pm$ 3.41	0.89 $\pm$ 0.41	3.2 $\pm$ 6.17	0.166
ALP (g/dL)	69.3 $\pm$ 60.9	63.4 $\pm$ 20.0	104.9 $\pm$ 150.5	0.142
AST (IU/dL)	33.2 $\pm$ 61.9	26.6 $\pm$ 20.9	72.8 $\pm$ 151.3	0.106
ALT (IU/dL)	34.5 $\pm$ 42.0	29.2 $\pm$ 23.3	67.3 $\pm$ 9.09	0.033
Size (mm)	16.1 $\pm$ 8.20	14.3 $\pm$ 6.3	26.7 $\pm$ 10.0	< 0.001
Size >15 mm	45 (69.2)	17 (48.6)	28 (93.3)	< 0.001
Location				0.705
Fundus	40 (61.5)	21 (60.0)	20 (66.7)	
Body	18 (27.7)	10 (28.6)	8 (26.7)	
Neck	3 (6.0)	2 (4.2)	1 (1.5)	
No. of polyps				0.534
Multiple	13 (20.0)	8 (22.9)	5 (16.7)	
Solitary	52 (80.0)	27 (77.1)	25 (83.3)	
Hyperechoic spots				0.912
No	56 (86.2)	30 (85.7)	26 (86.7)	
Yes	9 (13.8)	5 (14.3)	4 (13.3)	
Echogenicity				0.180
Hyperechoic	34 (52.3)	21 (60.0)	13 (43.3)	
Hypoechoic or isoechoic	31 (47.7)	14 (40.0)	17 (56.7)	
Echo pattern				0.016
Homogeneous	30 (46.2)	21 (60.0)	9 (30.0)	
Inhomogeneous	35 (53.8)	14 (40.0)	21 (70.0)	
Sonographic surface pattern				0.135
Smooth surface	45 (69.2)	27 (77.1)	18 (60.0)	
Nodular surface	20 (30.8)	8 (22.9)	12 (40.0)	

BMI: Body mass index; RUQ: Right upper quadrant; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine transaminase; Obesity: BMI higher than 25 kg/m².

determine the predictive values for neoplastic PLGs. We evaluated a total of 210 cases of resected GB polyps larger than 10 mm in size and we found that an older age (> 65 years), a history of DM and a large size were the significant predictive values for neoplastic PLGs. We also found that an older age (> 65 years), a history of DM and a large size were significant predictive values for malignant PLGs.

In our study, older patients more than 65 years showed a statistical relation with neoplastic or malignant PLGs, as compared to that of the younger patients ($P = 0.021$, $P = 0.005$, respectively). This result corresponds with previous studies about the correlation between age and neoplastic PLGs^[12,13,18,27,28]. With this background, we tried to determine a more detailed correlation of age with the

Table 4 Results of the multivariate logistic regression analysis for the factors that were significantly associated with the malignant gallbladder polyps for the 65 neoplastic polypoid lesions of the gallbladder on univariate analysis

	Hazard ratio	95% CI	<i>P</i> -value
Age $\geq$ 65 yr old	4.97	1.58-15.61	0.005
Gender, male	1.19	0.38-3.73	0.764
DM	6.13	1.98-18.94	0.001
ALT level	1.01	0.99-1.02	0.075
Polyp size > 15 mm	20.55	4.25-99.39	< 0.001
Inhomogeneous echo pattern	1.60	0.50-5.09	0.418

DM: Diabetes mellitus; ALT: Alanine aminotransferase.

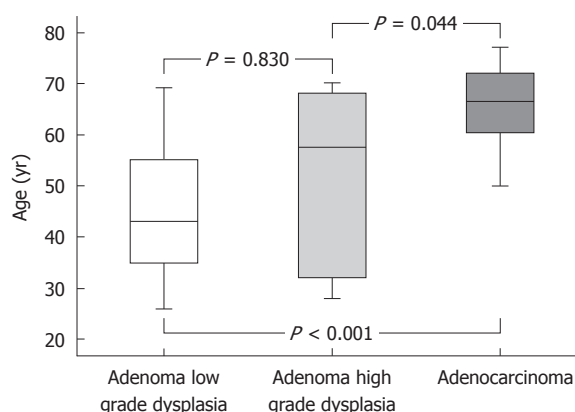


Figure 3 The age distribution according to the pathology subgroups with neoplastic polypoid lesions of the gallbladder.

subdivided groups among the neoplastic PLGs. According to pathologic results, the neoplastic PLGs were sorted into three subgroups; adenomatous polyp with low grade dysplasia, adenomatous polyp with high grade dysplasia and adenocarcinomas. We then compared the mean ages of each subgroup. After this detailed analysis, we found out a trend for a stepwise increase of mean age in the different neoplastic groups.

A new finding we discovered in this study was the relationship between DM and neoplastic polyps. Our results showed that patients with DM have a strong probability of having neoplastic and malignant polyps, as compared to that of the patients without DM, on univariate ($P < 0.001$, $P < 0.001$, respectively) and multivariate analyses, which were adjusted by age and gender (OR = 2.64, $P = 0.021$, and OR = 6.13, $P = 0.001$, respectively). There has been one document which reported the relation between diabetes and gallbladder cancer^[29]. But the exact mechanism or pathogenesis is not known. There have been a few reports that have found DM or hyperglycemia to be an independent risk factor for gastrointestinal or endocrine malignancies, such as colorectal^[30] or pancreatic cancers^[31]. Some recent researchers have proposed that the insulin resistance associated with hyperinsulinemia plays an important role as an oncogenic factor^[32,33]. According to many etiologic studies, it has become evident that the insulin-like growth fac-

tor (IGF) system plays a permissive role in cancer development and tumor progression^[34-38]. But, none of them mentioned any evidence of the IGF-I receptor pathway being involved in the development of gallbladder cancer. So we think that well designed trials are warranted in order to prove that this IGF signal pathway system plays a leading role in developing gallbladder cancer.

We found that the size of polyps (≥ 15 mm) is a powerful predictor for neoplastic polyps (OR = 4.94, $P < 0.001$). There was also a similar trend for malignant polyps (OR = 20.55, $P < 0.001$). Many studies have reported on the size criteria of PLGs as one of the predictive values for neoplastic lesions. The majority of them insisted that a size of gallbladder polyps more than 10 mm may be the most reliable predictor of malignant neoplasm^[12,13,18,27,28]. In a retrospective analysis of 354 subjects with resected PLGs, the authors suggested increasing the size criteria for cholecystectomy from 10 to 12 mm^[39]. Our study result showed a larger size than the previous noted criteria because small polyps less than 10 mm were not included in the analysis.

For the sonographic findings, solitary polyps ($P = 0.001$), an inhomogeneous echo pattern ($P = 0.019$) and a nodular surface pattern ($P < 0.001$) had a significant correlation with neoplastic PLGs on univariate analysis. However, only one variable, the nodular surface pattern, showed borderline statistical correlation with neoplastic polyps on the multivariate analysis. In addition, a nodular surface pattern did not show statistical significance with malignant polyps. The other sonographic parameters failed to show correlation with neoplastic or malignant PLGs. Many sonographers and endosonographers have recently tried to determine the sonographic characteristics that can reliably predict premalignant polypoid lesions in the gallbladder^[20,21,23,40]. They have suggested various sonographic findings as having predictive value for neoplastic lesions; the echo pattern, marginal irregularity, the shape, solitary lesion and preservation or loss of the GB wall layer structure. In spite of vigorous efforts to standardize these ultrasonographic features, inter-observer discrepancy is still the main concern to utilize these values to differentiate malignant polyps from benign polyps.

On the contrary, among 110 cases, which were lower risk groups for neoplastic polyps, such as those younger than 65 years old, those without DM and those with polyps less than 15 mm in sonographic diameter, 15 cases (13.6%) were reported as neoplastic polyps and the remaining 95 cases (86.4%) were non-neoplastic polyps.

The major limitations of this study include the following; first, this is not prospective study, rather, it is a cross-sectional study. There was no additional follow up data about the unresected PLGs more than 10 mm in size. However, because this study included patients who were consecutively enrolled during the study period, we could rule out a common selection bias. To the best of our knowledge, this study is the largest study that has enrolled patients with pathologically confirmed PLG larger than 10 mm in size. Thus, this data might be valuable when

making decisions on how to manage such patients with PLGs.

In conclusion, among patients with PLGs more than 10 mm in size, the higher risk groups, such as elderly patients who are more than 65 years, those with DM and those with a large sized polyp (≥ 15 mm) should be recommended cholecystectomy more seriously than other groups.

COMMENTS

Background

Some neoplastic polypoid lesions of the gallbladder (PLGs) including early cancer show similar appearances to the non-neoplastic PLGs. But there have been no definite guidelines except size criteria (more than 10 mm diameter) for the recommendation of surgical resection.

Research frontiers

Many studies have investigated the relationship between the neoplastic nature of PLGs and their morphological characteristics such as the number of polyps, the polyp shape, the diameter of the largest polyp, the echo level and internal echo pattern, and the polyp margin. But previously published documents showed a lack of case number, pathologic results, and long term follow up data. Also reports about the relationship between other clinical parameters and neoplastic PLGs were rare.

Innovations and breakthroughs

The authors performed the study using the consecutively enrolled pathologic data of patients with PLGs more than 10 mm in size to eliminate selection bias. This study demonstrated old age and diabetes history are added to the size criteria for predictive values of neoplastic PLGs for the decision about surgical resection.

Applications

Among patients with PLGs more than 10 mm in size considering surgical resection, the higher risk groups such as elderly patients who are more than 65 years, those with diabetes mellitus (DM) and those with a large sized polyp (≥ 15 mm) should be recommended cholecystectomy more seriously than other groups.

Terminology

Neoplastic PLGs: PLGs which have the features of the neoplasm including adenoma and adenocarcinoma. Non-neoplastic PLGs: PLGs which do not have the features of the neoplasm including cholesterol polyps, adenomyomatosis and inflammatory polyps.

Peer review

The authors described that older age, DM and polyp size > 15 mm were independent predictors of neoplasia as well as malignancy. Over all, this paper is well written, concise and information.

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Topical application of glycyrrhizin preparation ameliorates experimentally induced colitis in rats

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cytokine-induced neutrophil chemoattractant-2, and monocyte chemoattractant protein-1 in the inflamed mucosa. Furthermore, GL-p inhibited the oxidative activity of mucosal and purified MPO.

CONCLUSION: GL-p enema has a therapeutic effect on experimental colitis in rats and may be useful in the treatment of UC.

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Key words: Glycyrrhizin; Colitis; Dextran sodium sulfate; Ulcerative colitis; Cytokine; Chemokine; Protein array; Myeloperoxidase; Enema; Carboxymethylcellulose

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Abstract

AIM: To examine the efficacy of glycyrrhizin preparation (GL-p) in the treatment of a rat model of ulcerative colitis (UC).

METHODS: Experimental colitis was induced by oral administration of dextran sodium sulfate. Rats with colitis were intrarectally administered GL-p or saline. The extent of colitis was evaluated based on body weight gain, colon wet weight, and macroscopic damage score. The expression levels of pro-inflammatory cytokines and chemokines in the inflamed mucosa were measured by cytokine antibody array analysis. The effect of GL-p on myeloperoxidase (MPO) activity in the inflamed mucosa and purified enzyme was assayed.

RESULTS: GL-p treatment significantly ameliorated the extent of colitis compared to sham treatment with saline. Cytokine antibody array analysis showed that GL-p treatment significantly decreased the expression levels of pro-inflammatory cytokines and chemokines, including interleukin (IL)-1 β , IL-6, tumor necrosis factor- α ,

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammation of the large intestine, which is characterized by pronounced infiltration of neutrophils into colonic lesions with hyperemia, hemorrhage, and ulcerations. Its etiology is not fully understood but immunological mechanisms seem to be involved^[1,2]. Increased pro-inflammatory mediators, including cytokines and chemokines, are observed in patients with colitis^[3]. An efficacious treatment strategy comprises controlling the expression and neutralizing the function of these mediators. Agents such as aminosalicylates, corticosteroids, and immunosuppressants inhibit the

production of these factors and are used in the treatment of UC; for example, antibodies that target tumor necrosis factor (TNF)- α are clinically efficacious to neutralize TNF- α actions^[1,2]. However, these agents may cause serious adverse effects, such as agranulocytosis, severe infections, osteoporosis, and malignant lymphoid tumors.

Glycyrrhizin (GL), a natural compound extracted from the roots of the Chinese herb *Glycyrrhiza glabra*, has been used for several centuries in traditional Chinese medicine. Its preparation (GL-p) has been clinically used as an anti-allergic agent and also as treatment for chronic hepatitis for more than 30 years^[4]. GL-p has useful pharmacological properties, such as anti-inflammatory, immunomodulatory, and cytoprotective effects^[5]. Furthermore, treatment with GL-p is rarely accompanied by severe adverse effects, even during long-term use. However, the efficacy of GL-p in the treatment of other inflammatory conditions including UC is not well known.

Several models of experimentally induced colitis have been developed to investigate the mechanisms of inflammation and immunological disturbances^[6]. Among them, an animal model with dextran sulfate sodium (DSS)-induced colitis exhibits several symptoms similar to those seen in human UC; therefore, they are considered reliable for studying the pathogenesis of UC^[6,7].

To confirm the hypothesis that GL-p ameliorates colitis through its pharmacological effects, we have conducted experiments to investigate the therapeutic efficacy of GL-p for DSS-induced colitis in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (approximately 250 g, 8 wk old) were used for the experiments. All rats were housed in specific pathogen-free conditions in the animal facility of Gunma University and fed standard laboratory chow and tap water *ad libitum*. This study was approved by the Animal Care and Experimentation Committee at Gunma University.

Induction of experimental colitis

To induce colitis, rats were orally administered 3% solution of DSS (molecular weight, 5000; Wako Pure Chemical Industries, Ltd., Osaka, Japan) *via* drinking water for 7 d (from day 0 to day 6).

Treatment design

GL-p is commercially supplied by Minophagen Pharmaceutical (Tokyo, Japan) as a solution (Stronger Neo-Minophagen C®), which contains 2 mg GL monoammonium, 1 mg L-cysteine, and 20 mg glycine per mL in physiological saline solution. Rats in the GL-p and GL groups were respectively administered 1 mL GL-p or 0.2% GL solution transanally under diethyl ether anesthesia, once daily for four consecutive days (day 3 to day 6). Control rats were administered 1 mL saline. Body weight was measured throughout the experiments. All rats were killed on day 7 under excess diethyl ether anesthesia.

Assessment of mucosal damage

Body weight gain was calculated by subtracting the body weight at the beginning of the treatment from that at autopsy. An 8-cm long biopsy specimen of the distal colon was resected at autopsy and opened by longitudinal incision. The wet weight of this biopsy specimen was measured followed by observation of the gross appearance of the mucosa. Mucosal damage was measured and macroscopically scored on a scale of 0 to 10 according to the following criteria^[8]: 0, no damage; 1, hyperemia without ulcers; 2, hyperemia and thickening of the bowel wall without ulcers; 3, one ulcer without thickening of the bowel wall; 4, two or more sites of ulceration and inflammation; 5, two or more major sites of ulceration and inflammation or one site of ulceration and inflammation extending > 1 cm along the length of the colon; 6-10, one point being added for each additional centimeter of involvement beyond an initial 2 cm. Tissue specimens were kept at -80°C until cytokines were evaluated.

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to the modified method of Bradley *et al.*^[9]. The mucosal scrapings were homogenized with a Diox 600 homogenizer (Heidolph, Germany) in 1 mL buffer that contained 0.5% hexadecyltrimethylammonium bromide and 50 mmol/L potassium phosphate (pH 6.0). The homogenates were sonicated for 10 s, freeze-thawed three times, and centrifuged at 40 000 $\times g$ for 15 min at 4°C. From each sample, 100 μ L was added to 2.9 mL 50 mmol/L phosphate buffer (pH 6.0) that contained 0.167 mg/mL o-dianisidine hydrochloride and 0.0005% hydrogen peroxide. MPO activity was measured colorimetrically using a spectrometer with a change of absorbance of 460 nm during a 30-min interval at 25°C. One unit of MPO activity was defined as 1 mmol H₂O₂ broken down to H₂O and results were expressed as units per gram mucosal tissue. The MPO activities in the supernatant of mucosal homogenate and in the purified human MPO enzyme (Sigma-Aldrich Japan, Tokyo, Japan) were measured in the presence and absence of various amounts of GL-p.

Measurement of cytokines and chemokines using antibody array

The Cytokine Array (Raybiotech Inc., Norcross, GA, USA)^[10] was used to detect 19 different cytokines and chemokines in the supernatant of homogenized colonic mucosal scrapings according to the manufacturer's recommended protocol. After the membranes were exposed to X-ray film (GE Healthcare Bioscience Co. Ltd.), the exposed films were digitized and the relative cytokine levels were compared by densitometrical analysis using ImageJ ver. 1.38x software (National Institute of Health, Rockville Pike, Bethesda, MD, USA). The relative cytokine levels were obtained by subtracting the background staining and normalizing from the positive controls on the same membrane.

Statistical analysis

All data are presented as mean $\pm$ SE. Student's *t* test was used for comparison between the data in the two groups. One-way ANOVA followed by Tukey's *post hoc* test was used to analyze the data for multiple groups. For evaluation of the damage score, the non-parametric Kruskal-Wallis test followed by the Steel-Dwass test was used. $P < 0.05$ was considered statistically significant. KyPlot 5.0 (KyensLab. Inc., Tokyo, Japan) was used for the statistical analyses.

RESULTS

Effects of GL-p enema on experimental colitis

After treatment with 3% DSS for 7 d, all the rats developed symptoms of colitis. Diarrhea was first observed on day 4 after the onset of treatment, followed by rectal bleeding and body weight loss. Compared to the control group, the GL-p and GL groups experienced significantly increased body weight gain ($P < 0.05$), decreased colon wet weight ($P < 0.05$), and reduced macroscopic damage score ($P < 0.05$) (Figure 1). Significant differences between GL-p and GL groups were not detected (Figure 1).

Effects of sodium carboxymethylcellulose accompanied with GL-p on experimental colitis

To determine whether the addition of carboxymethylcellulose (CMC) as a viscosity modifier augments the therapeutic effects of GL-p enema, we compared the group treated with GL-p alone to those treated with a combination of GL-p and CMC. The addition of 0.5% CMC seemed to increase body weight gain and significantly decrease the colon wet weight compared to GL-p alone ($P < 0.01$), whereas addition of CMC did not result in any changes in the macroscopic damage score (Figure 2).

Cytokine antibody array analysis

Using cytokine antibody array analysis, the 19 cytokine levels between the GL-p and control groups were compared (Figure 3). The levels of pro-inflammatory cytokines including interleukin (IL)-1 β , IL-6 and TNF- α were significantly decreased in the GL-p group, and those of chemokines, such as cytokine-induced chemoattractant (CINC)-2 and -3, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-3 α , were also significantly inhibited in the GL-p group. Among Th1 cytokines, interferon (IFN)- γ was not significantly inhibited. Among Th2 cytokines, IL-4 and IL-6 decreased while IL-10 did not change. The levels of leptin, tissue inhibitor of metalloproteinase (TIMP)-1, fractalkine, and ciliary neurotrophic factor (CNTF) were decreased in the GL-p group.

Effects of GL-p on MPO activity

To evaluate the effect of GL-p on the number of neutrophils infiltrating the colon, levels of mucosal MPO activity were measured. Rats treated with GL-p showed a significantly low level of mucosal MPO activity compared to con-

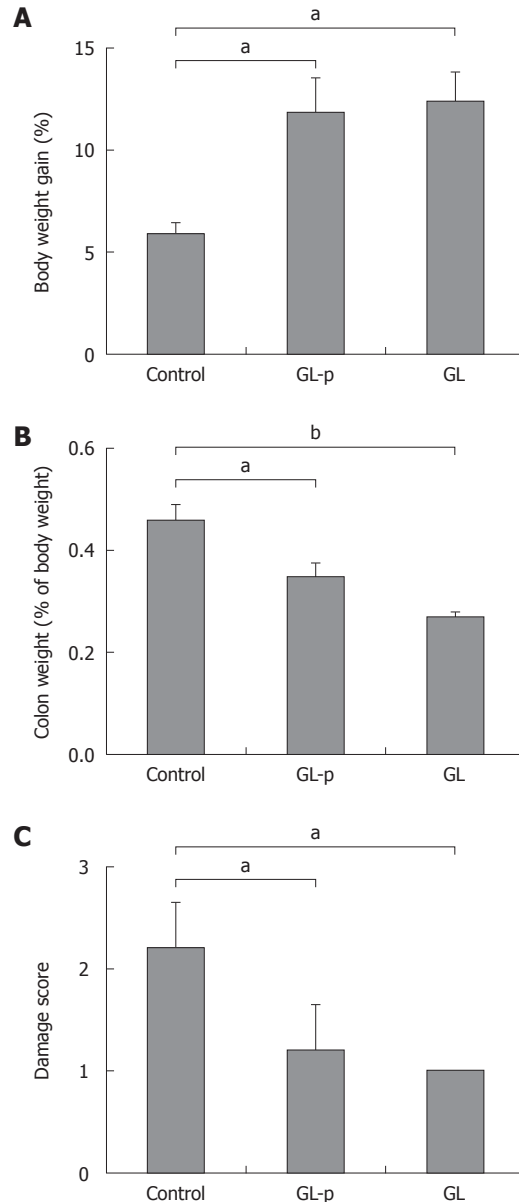


Figure 1 Effects of glycyrrhizin preparation and glycyrrhizin treatments on body weight gain (A), colon wet weight (B), and macroscopic damage score (C) in rats with dextran sulfate sodium-induced colitis. Data are expressed as the mean $\pm$ SE of five rats. GL-p: Glycyrrhizin preparation. ^a $P < 0.05$, ^b $P < 0.01$.

trol rats ($P < 0.05$) (Figure 4A). We conducted *in vitro* experiments to assess the presence of an inhibitory effect of GL-p on MPO activity, and found that GL-p dose-dependently inhibited MPO activity in mucosal tissue (Figure 4B), as well as purified human enzyme (Figure 4C).

DISCUSSION

In the present study, we showed that GL-p ameliorated the extent of DSS-induced colitis in rats. We used cytokine antibody array analyses and revealed that GL-p reduced the level of pro-inflammatory cytokines and chemokines in the colonic mucosa. Furthermore, GL-p was shown to inhibit MPO activity.

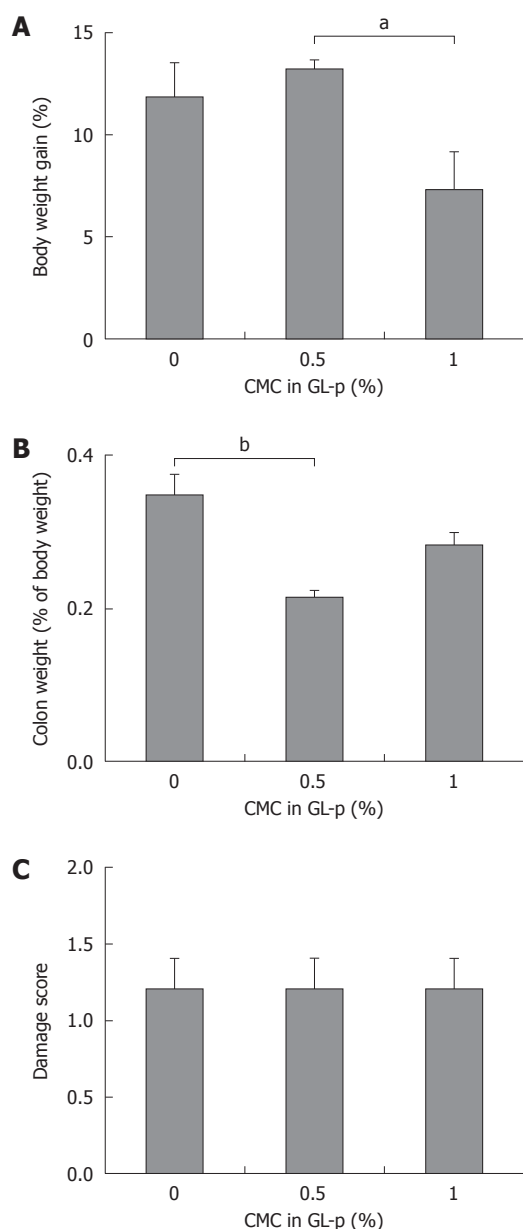


Figure 2 Effects of the addition of sodium CMC to glycyrrhizin preparation on body weight gain (A), colon wet weight (B), and macroscopic damage score (C) in rats with dextran sulfate sodium-induced colitis. Data are expressed as the mean $\pm$ SE of five rats. ^a $P < 0.05$, ^b $P < 0.01$. CMC: Carboxymethyl cellulose.

We demonstrated that GL-p administration ameliorated the production of pro-inflammatory cytokines and chemokines, such as IL-1 β , TNF- α , IL-6, CINC-2 and -3, MCP-1, MIP-3 α , TIMP-1, fractalkine, CNTF, leptin, and GM-CSF. The relevance of pro-inflammatory cytokines and chemokines to the pathophysiology of chronic inflammation in inflammatory bowel disease (IBD) has recently been elucidated, and their manipulation has successfully reduced disease severity and maintained remission. IL-1 β has been well characterized as a potent inflammatory cytokine that is produced by inflammatory and mucosal epithelial cells during colonic inflammation^[11]. Its neutralization with antibodies suppresses the development

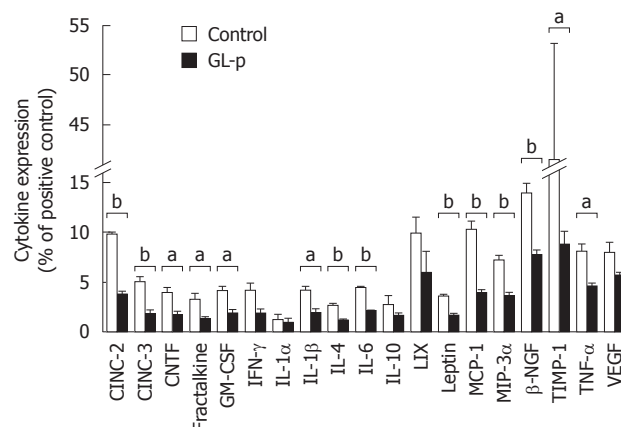


Figure 3 Expressions of cytokines and chemokines in the extract of colonic mucosa by cytokine antibody array analysis. GL-p: Glycyrrhizin preparation; CINC: Cytokine-induced chemoattractant; CNTF: Ciliary neurotrophic factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; IL: Interleukin; LIX: Lipopolysaccharide-induced CXC chemokine; MCP: Monocyte chemoattractant protein; MIP: Macrophage inflammatory protein; NGF: Nerve growth factor; TIMP: Tissue inhibitor of metalloproteinase; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor. $n = 3$ in each group. ^a $P < 0.05$, ^b $P < 0.01$.

of DSS-induced colitis^[12]. IL-6 is a pleiotropic cytokine that exerts its pro-inflammatory effects mainly by means of its soluble IL-6 receptor. The anti-IL-6 receptor monoclonal antibody abrogates murine colitis^[13]. TNF- α is a key inflammatory cytokine produced mainly from macrophages and is a target of IBD treatment. Anti-TNF- α antibody and soluble TNF- α receptor are used clinically with significant benefits. Collectively, these findings suggest the efficacy of GL-p in the treatment of human UC.

GL-p used in this study was a combination of GL with 2% of glycine and 0.4% of L-cysteine. A diet that contains 5% glycine has recently been reported to prevent colitis in rats, which is chemically induced by DSS or 2,4,6-trinitrobenzene sulfonic acid (TNBS)^[14]. Glycine inhibits the induction of pro-inflammatory cytokines and chemokines, such as IL-1 β , TNF- α , CINC and MIP-2. On the contrary, dietary L-cysteine supplementation is also reported to have a therapeutic effect on DSS-induced porcine colitis^[15]. However, no significant therapeutic differences were observed between GL alone and GL-p in the present study (Figure 1). Although the difference in the experimental settings, such as the route of administration, may be relevant to this contradiction, the precise reasons for this are unknown. One possibility is that the effect of amino acids in GL-p was masked by that of GL in our experimental settings. To evaluate this possibility, further experiments including a control group treated with amino acids alone is required.

Our study demonstrated that GL was beneficial in the treatment of rat colitis if administered intrarectally (Figure 1). However, GL was not proven to be efficacious in a previous study^[16]. Although the beneficial effects of oral administration of Hange-shashin-to (HST), a combination of seven herbs^[16], on DSS-induced colitis in rats

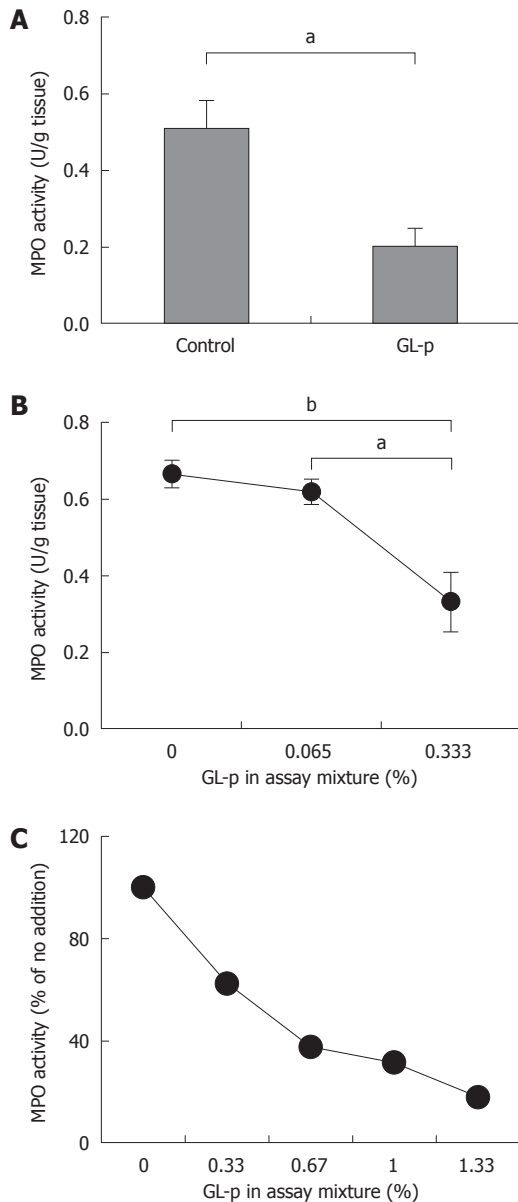


Figure 4 Glycyrrhizin preparation and myeloperoxidase activity. A: Effects of glycyrrhizin preparation (GL-p) treatment on myeloperoxidase (MPO) activity in the mucosa of rats with dextran sulfate sodium-induced colitis ($n = 5$ in each group); B: Effects of GL-p on MPO activity in the mucosal extract ($n = 5$ in each group); C: Effects of GL-p on MPO activity of purified human enzyme. ^a $P < 0.05$, ^b $P < 0.01$.

has been demonstrated, the previous study did not detect the effects of oral administration of a single constituent included in HST, such as GL. One possible explanation for the contradiction between their results and ours may be the difference in the type of experimental colitis used. They used a model of colitis induced by intracolonic instillation of TNBS, which is known as a model of Crohn's disease, and has distinct immunological features from those of the DSS-induced colitis that we used^[6]. This seems to have caused conflicting responses to GL administration, which suggests that Crohn's disease and UC should be distinguished at the point of clinical application of GL treatment. Another possibility is the

differences in dose and route of administration. They administered an oral dose of 2.67 or 5.37 mg/kg GL. We administered a dose of 8 mg/kg (2 mg GL per rat weighing 250 g) intrarectally. We were subsequently able to deliver higher concentrations of GL to the inflamed colon compared to the previous study.

Regarding GL administration for colitis, Yuan *et al*^[17] recently have reported the results of experiments using colitis induced by acetic acid installation, which is a model of acute colitis^[6]. They found that GL reduced colonic injury with the suppression of nuclear factor- κ B, TNF- α , and intercellular adhesion molecule-1 in the affected mucosa. They administered GL at a dose of 40 mg/kg, which was five times greater than that in our study. Furthermore, they chose the intraperitoneal route for administration. Use of this route may raise the systemic concentration of GL sufficiently high to cause adverse effects such as severe hypertension, hypokalemia, and other signs of mineralocorticoid excess, because the incidence of adverse effects is dose-dependent^[4,5]. We administered GL and GL-p intrarectally to maintain low systemic concentrations of the agents despite high local concentrations^[18]. For clinical application, rectal administration is preferable as it may maximize quality of life by reducing the necessity for frequent visits to outpatient clinics to receive parenteral administration of GL-p.

We demonstrated that GL-p treatment decreased MPO activity in the inflamed mucosa (Figure 4A), which indicates the inhibition of neutrophil accumulation at the site of injury through GL-p administration. MPO is mainly produced by neutrophils that infiltrate the inflammatory sites, therefore, MPO activity in specimens is believed to reflect the number of infiltrating neutrophils^[9]. Furthermore, in this study, we demonstrated for the first time that GL-p suppressed MPO activity in a dose-dependent manner in experiments using the inflamed mucosa (Figure 4B), as well as purified human enzyme (Figure 4C). This appears to be a new mechanism in the therapeutic actions of this agent. Although the main function of MPO is to destroy phagocytosed microorganisms by strong oxidative species within the phagosome, the excessive amount and activation of MPO at inflamed sites may cause tissue injury by modification of lipids and proteins through reactive oxidative species^[19]. Therefore, modulation of MPO oxidation activity by GL-p has a therapeutic potential for inflammation.

In the treatment of UC, development of colorectal carcinoma is a serious complication. It is noticeable that long-term use of GL-p is effective in preventing development of hepatocellular carcinoma in patients with chronic hepatitis C^[20,21]. Amelioration of chronic inflammation is suggested to reduce the possibility of oncogenesis, therefore, GL-p enema may prevent the development of colon cancer.

In conclusion, our study suggests a therapeutic effect of GL-p enemas on experimental colitis in rats. Further studies are necessary to determine an effective administration strategy with higher efficacy and fewer adverse

effects in the treatment of patients with UC.

COMMENTS

Background

Ulcerative colitis (UC) affects many people worldwide. Its pathophysiology involves immunological mechanisms. Glycyrrhizin preparation (GL-p) may be useful in the treatment of UC because it has anti-inflammatory and immunomodulatory effects.

Research frontiers

Although a previous study has demonstrated that oral administration of a combination of seven herbs is beneficial in the treatment of colitis in rats, the effect of GL, one of its constituents, was not detected.

Innovations and breakthroughs

The study demonstrated that GL-p was beneficial in the treatment of rat colitis if administered intrarectally. GL-p decreased the expression of pro-inflammatory cytokines and inhibited myeloperoxidase activity.

Applications

The results of this study suggest a therapeutic effect of GL-p enemas on experimental colitis in rats. Further studies are required to apply this treatment in patients with UC.

Terminology

GL is a natural compound extracted from the roots of the Chinese herb *Glycyrrhiza glabra* and GL-p has been clinically used as an anti-allergic agent and as a treatment for chronic hepatitis in Japan.

Peer review

GL, an active ingredient from licorice root, has long been used for traditional medicinal purposes. Very little work on this subject has been described in the mainstream literature, and this work is a welcome addition. The authors have done a commendable job of comparing their study with the only (to my knowledge) previously published work in the field, primarily that of Yuan *et al.*

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Hyperbaric oxygenation promotes regeneration of biliary cells and improves cholestasis in rats

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did not modulate HGF or TGF β -1 mRNA expression levels.

CONCLUSION: HBO promoted regeneration of biliary ductal cells and improved postoperative cholestasis after a partial hepatectomy.

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Key words: Hyperbaric oxygenation; Liver regeneration; Partial hepatectomy; Cholestasis; Ballooning

Peer reviewer: Christopher Christophi, Professor and Head of The University of Melbourne Department of Surgery, Austin Hospital, Melbourne, 145 Studley Road, Victoria 3084, Australia

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Abstract

AIM: To investigate the effects of hyperbaric oxygenation (HBO) on regeneration of the biliary ductal system and postoperative cholestasis in hepatectomized rats.

METHODS: HBO was performed in Wistar rats daily starting 12 h after a 70% partial hepatectomy. Regenerated liver weight, serum parameters and the proliferating cell nuclear antigen labeling index of hepatocytes and biliary ductal cells were measured. Hepatocyte growth factor (HGF), c-Met and transforming growth factor (TGF) β -1 mRNA expression levels were analyzed by quantitative reverse transcription polymerase chain reaction.

RESULTS: HBO improved the postoperative serum levels of total bile acid but not transaminase levels. HBO promoted hepatocyte and biliary ductal cell proliferation. The hematoxylin and eosin-stained specimens revealed fewer ballooned hepatocytes and higher cell densities in the HBO group compared to the control group. HBO suppressed c-Met mRNA levels at 15 h but

INTRODUCTION

Extended hepatectomy and small graft liver transplantation can result in postoperative cholestasis and liver failure. Hepatectomized liver rapidly regenerates and its volume and function recover, but functional recovery lags behind quantitative recovery. Postoperative liver failure causes cholestasis; hence, it is important to improve cholestasis in order to reduce morbidity and mortality.

Hyperbaric oxygenation (HBO) has been used as a therapy in patients with carbon monoxide poisoning, decompression sickness and arterial gas embolism^[1]. Effects of HBO that have been reported include upregulation of growth factors^[2], down-regulation of inflammatory cytokines^[3] and increased angiogenesis^[4].

Studies have reported the effect of HBO on liver re-

generation after partial hepatectomy, but few studies have reported its effect on biliary ductal regeneration and cholestasis. In this study, we investigated the effects of HBO on the biliary system and postoperative cholestasis after a partial hepatectomy.

MATERIALS AND METHODS

Animals and surgery

All experiments were performed on 10-wk-old male Wistar rats ($n = 78$). Thirty-six rats were used in each group and preoperative data acquired from another 6 rats. Procedures were approved by the Review Committee on Animal Use of Gunma University, Maebashi, Japan. All the rats were housed under temperature- and light-controlled conditions with 12 h light and dark cycles. The rats were fed a standard rat diet and water as desired. Prior to the experiment, the rats were starved for 24 h with free access to water. Under ether anesthesia, a 70% partial hepatectomy was performed using the Higgins and Anderson technique^[5]. After the transverse laparotomy, the median and left lobes were ligated and resected. All the rats were allowed free access to the standard diet and water after the surgery. The rats were sacrificed at various time points (12, 15, 24, 48, 72, and 96 h after the partial hepatectomy) by puncture of the inferior vena cava and exsanguination under ether anesthesia. The regenerated livers were removed and either fixed in 20% formalin or frozen and stored at -80°C .

HBO

The rats in the HBO group were placed in a hyperbaric chamber (Model KHO-200; Kawasaki Engineering Co., Ltd., Hyogo, Japan) pressurized to 2 ATA with 100% oxygen for 60 min. HBO was initiated 12 h after the end of surgery and performed once a day until postoperative day 4. HBO was not performed postoperatively in the control group.

Regenerated liver weight/% body weight

The regenerated liver weight/% body weight ratio was calculated using the following equation: $100 \times A/B$, where A is the weight of the regenerated liver at the time of sacrifice and B is the rat body weight at the time of sacrifice.

Serum parameters

The degree of hepatic injury and biliary function was assessed by determining the serum levels of alanine aminotransferase (ALT) and total bile acid (TBA) using standard laboratory methods.

Hematoxylin and eosin staining

Formalin-fixed liver specimens were embedded in paraffin, and 3- μm sections were prepared. Hematoxylin and eosin (HE) staining was performed according to standard procedures. Hepatocytes were counted in random high power fields (HPFs) without a visible Glisson's sheath or

central vein ($\times 400$). The cell density was expressed as the mean number of hepatocytes per HPF.

Immunohistochemistry

Proliferating cell nuclear antigen (PCNA) expression was immunohistochemically detected using a monoclonal antibody against PCNA [dilution: 1:400, horseradish peroxidase-labeled, DAKO, PC10, (Code No. M0879)]. PCNA-positive cells were counted in random HPFs ($\times 400$). The PCNA-labeling index was expressed as the percentage of PCNA-positive cells/total number of cells. Hepatocytes and biliary ductal cells were counted.

RNA isolation and cDNA synthesis

Total RNA was extracted from freshly frozen liver using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity of isolated RNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). Template cDNA was synthesized from 13.5 μL of total RNA using an Omniscript reverse transcriptase kit (Qiagen), a random primer (hexadeoxyribonucleotide mixture) (TaKaRa, Shiga, Japan), and a ribonuclease inhibitor (porcine liver) (TaKaRa). Total RNA was reverse transcribed using 4 U of Omniscript reverse transcriptase in a reaction volume of 20 μL (60 min at 37°C , 5 min at 93°C , then placed on ice). The resultant cDNA samples were stored at -20°C until analysis.

Quantitative reverse transcription polymerase chain reaction analysis of hepatocyte growth factor, c-Met, and transforming growth factor β -1 mRNA expression levels

Quantitative reverse transcription-polymerase chain reaction (PCR) analyses were performed using the ABI Prism 7000 Sequence detection system (Applied Biosystems, CA, USA). The standard reaction volume was 20 μL and contained $1 \times$ SYBR Green PCR Master Mix (Applied Biosystems), 2.0 μL of cDNA template, and 0.5 $\mu\text{mol/L}$ each of the forward and reverse primers. The initial PCR denaturation step was performed for 10 min at 95°C followed by 45 cycles of 15 s at 95°C (denaturation), 10 s at 60°C (annealing), and 30 s at 72°C (extension). All reactions were performed in duplicate. The data were adjusted using the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and expressed as a ratio of the GAPDH levels. The primers for hepatocyte growth factor (HGF) and transforming growth factor (TGF) β -1 were used as described in previously published assays^[6], and the primers for c-Met and GAPDH were designed using Primer 3 (primer3_www.results.cgi) with sequences from Ensembl Genome Browser (<http://www.ensembl.org/index.html>) (Table 1).

Statistical analysis

All values are expressed as mean $\pm$ SD. Statistical significance was determined using the post-hoc test of Bonferroni-Dunn. $P < 0.05$ was considered statistically significant.

Table 1 Primer sequence for reverse transcription polymerase chain reaction

Gene		Primer sequence	Annealing temperature (°C)
HGF	Sense	5'-TTATGGGGAATGAGAAATGC	60
	Antisense	5'-TCGAACAAAAATACCAGGAC	
c-Met (ENSRNOT00000009662)	Sense	5'-CAGCGGCAATTCTAGACACA	60
	Antisense	5'-CTGAAGCTGCTTGTCACTCG	
TGF β -1	Sense	5'-ATGACATGAACCGACCCTTC	60
	Antisense	5'-TGTGTTGGTTGTAGAGGGCA	
GAPDH (ENSRNOT00000002652)	Sense	5'-AGACTATGGATGGCCCTCT	60
	Antisense	5'-GGTAGGAACACGGAAGACCA	

HGF: Hepatocyte growth factor; TGF: Transforming growth factor.

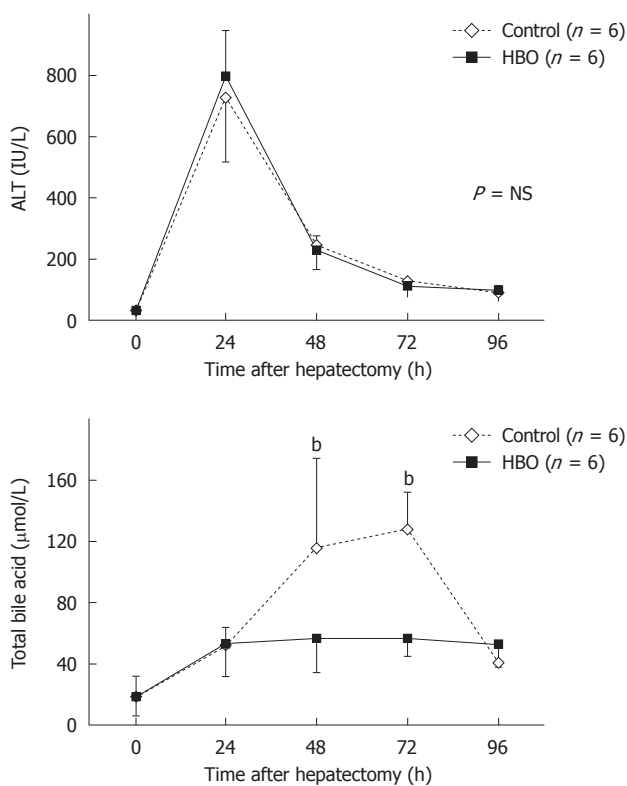


Figure 1 Serum parameters after hepatectomy. No significant difference was observed in the postoperative serum alanine aminotransferase (ALT) levels between the 2 groups. Total bile acid levels at 48 and 72 h after hepatectomy were significantly lower in the hyperbaric oxygenation (HBO) group than in the control group. Results are expressed as mean $\pm$ SD of $n = 6$ for each period in each group. NS: No significant. ^b $P < 0.01$.

RESULTS

Liver injury and biliary function after the partial hepatectomy

Blood parameters were measured at various time points. ALT levels peaked at 24 h after the partial hepatectomy in the control and HBO groups and then decreased rapidly (Figure 1). No significant differences were observed between the groups at any time point as the serum ALT level decreased. Serum TBA levels increased and peaked at 72 h (Figure 1); these were decreased by HBO. Signifi-

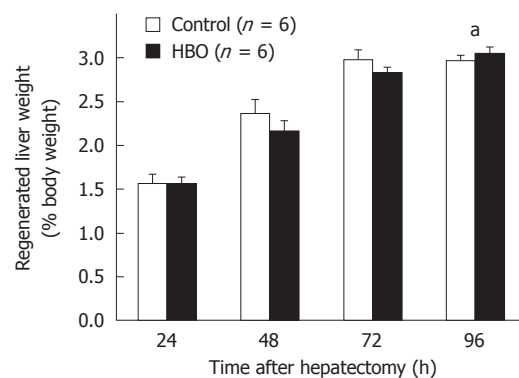


Figure 2 Regenerated liver weight (% body weight). In both groups, the percentage weight of the regenerated liver increased daily after hepatectomy. At 96 h after hepatectomy, the percentage weight was higher in the hyperbaric oxygenation (HBO) group than in the control group. Results are expressed as mean $\pm$ SD of $n = 6$ for each period in each group. ^a $P < 0.05$.

cant differences were observed in TBA levels at 48 h and 72 h after the partial hepatectomy. These data indicate that HBO improved the postoperative biliary function.

Liver regeneration after the partial hepatectomy

Data in Figure 2 reveal that the regenerated liver weight/% body weight increased daily after the partial hepatectomy. At 96 h, the regenerated liver weight ratio was higher in the HBO group than in the control group. The PCNA labeling index of the hepatocytes was higher at 48 and 72 h and lower at 24 h in the HBO group than in the control group (Figure 3A). The PCNA labeling index of the biliary ductal cells peaked at 48 h in the HBO group and was significantly higher in the HBO group than in the control group (Figure 3B). Combined, these data indicate that biliary ductal cell proliferation was greater in the HBO group than in the control group.

HE staining after the partial hepatectomy

An edematous cytoplasm and ballooning hepatocytes were observed in the regenerated livers of the control group at 96 h after surgery; however, these findings were not observed in the HBO group (Figure 4). The cell density of the hepatocytes was higher in the HBO group at

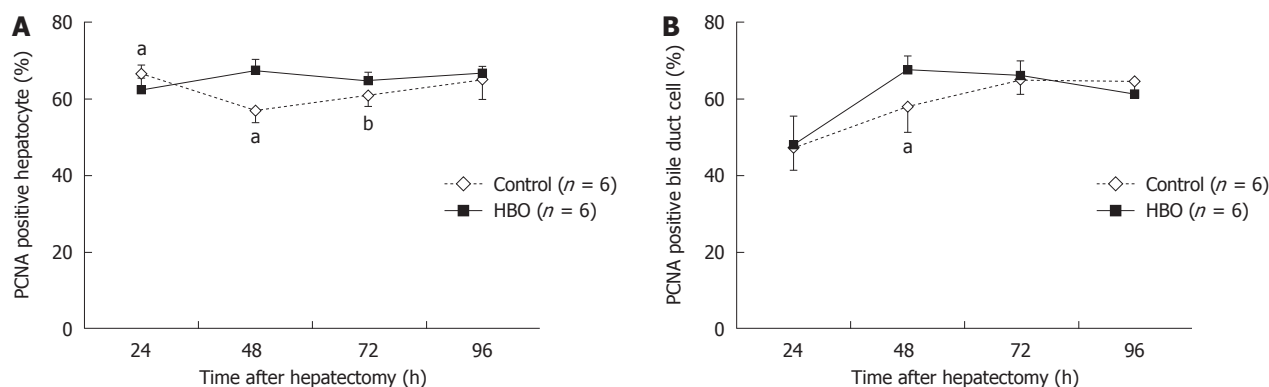


Figure 3 Percentage of proliferating cell nuclear antigen-positive cells in both groups at 24, 48, 72 and 96 h after hepatectomy. A: The percentage of proliferating cell nuclear antigen (PCNA)-positive hepatocytes was higher at 48 and 72 h and lower at 24 h in the hyperbaric oxygenation (HBO) group than in the control group. The percentage of PCNA-positive biliary ductal cells in the HBO group was higher at 48 h compared with the control group; B: The proliferation of biliary ductal cells was increased in the HBO group, with an early peak at 48 h. Results are expressed as mean $\pm$ SD of $n = 6$ for each period in each group. ^a $P < 0.05$, ^b $P < 0.01$.

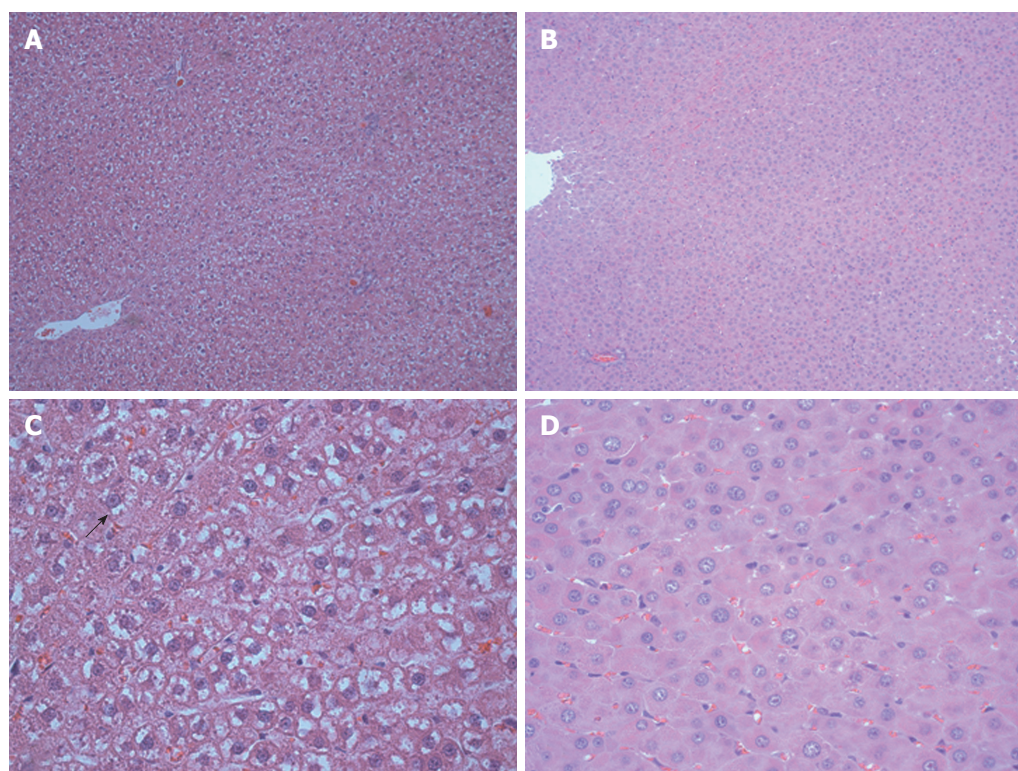


Figure 4 Hematoxylin and eosin staining of the liver slices at 96 h after hepatectomy. Control group (A: 100 $\times$ magnification, C: 400 $\times$). Hyperbaric oxygenation (HBO) group (B: 100 $\times$, D: 400 $\times$). In the control group, the regenerating livers were edematous and showed ballooned hepatocytes (C, arrow). In addition, the number of hepatocytes per high power field was lower in the control group than in the HBO group.

72 and 96 h (Figure 5). These findings suggest that HBO inhibited cell edema and protected the hepatocytes during liver regeneration.

Expression of HGF, c-Met and TGF β -1 mRNA

The expression of HGF and TGF β -1 mRNA peaked at 15 h in both groups and no significant differences in the mRNA levels of the HBO and control groups were observed. While the mRNA expression of c-Met increased and peaked at 15 h in both the groups, expression was significantly suppressed in the HBO group (Figure 6).

DISCUSSION

The goal of this study was to determine the effect of HBO on liver regeneration, particularly in biliary system restoration and improvement of postoperative cholestasis.

HBO involves the inspiration of a high concentration of oxygen in a pressure chamber at pressures higher than 1 ATA. During HBO, the partial pressure of oxygen increases, thereby increasing oxygen delivery throughout the body. The beneficial effects of HBO have been reported in ischemia-reperfusion injuries^[6-8], acute liver fail-

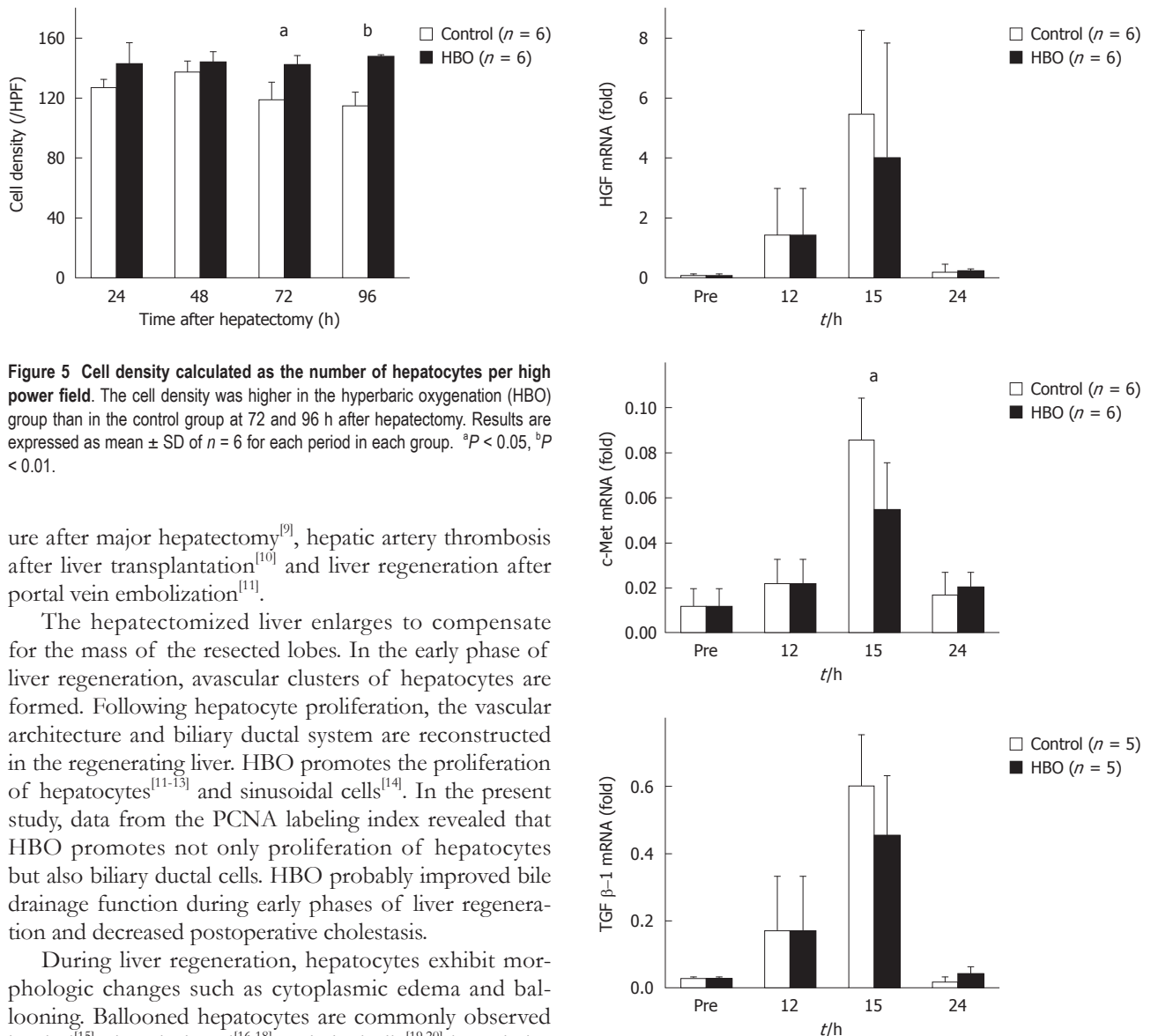


Figure 5 Cell density calculated as the number of hepatocytes per high power field. The cell density was higher in the hyperbaric oxygenation (HBO) group than in the control group at 72 and 96 h after hepatectomy. Results are expressed as mean $\pm$ SD of $n = 6$ for each period in each group. ^a $P < 0.05$, ^b $P < 0.01$.

ure after major hepatectomy^[9], hepatic artery thrombosis after liver transplantation^[10] and liver regeneration after portal vein embolization^[11].

The hepatectomized liver enlarges to compensate for the mass of the resected lobes. In the early phase of liver regeneration, avascular clusters of hepatocytes are formed. Following hepatocyte proliferation, the vascular architecture and biliary ductal system are reconstructed in the regenerating liver. HBO promotes the proliferation of hepatocytes^[11-13] and sinusoidal cells^[14]. In the present study, data from the PCNA labeling index revealed that HBO promotes not only proliferation of hepatocytes but also biliary ductal cells. HBO probably improved bile drainage function during early phases of liver regeneration and decreased postoperative cholestasis.

During liver regeneration, hepatocytes exhibit morphologic changes such as cytoplasmic edema and ballooning. Ballooned hepatocytes are commonly observed in viral^[15], drug-induced^[16-18] and alcoholic^[19,20] hepatic injuries and non-alcoholic steatohepatitis^[21,22]. Hepatocytes are swollen in biopsies of orthotopic liver grafts after transplantation. Ballooning is associated with, but not directly caused by, bile retention. One of the pathogenetic factors of ballooned hepatocytes is ischemic damage. In a rat steatotic liver model, swollen hepatocytes caused abnormal microcirculation, manifested as reduced sinusoidal density^[23]. The edematous state of hepatocytes can cause obstruction of the sinusoids and biliary ducts, secondary cholestasis and circulatory failure in the liver. HBO increases oxygen delivery and improves the ischemic state of the regenerated liver, breaking the cycle of hepatocyte ballooning and abnormal microcirculation. Our results suggest that HBO suppresses the edematous state of hepatocytes, accelerates bile drainage and improves postoperative cholestasis.

The present study revealed that HBO did not lower postoperative ALT levels. One study has suggested that HBO reduces serum ALT levels after a partial hepatectomy^[14], while others have revealed no significant differences in ALT levels during liver regeneration^[12,13,24]. The

Figure 6 Expressions of hepatocyte growth factor, c-Met and transforming growth factor β -1 mRNA in the liver tissues determined at various time points. Results are expressed as mean $\pm$ SD. Hepatocyte growth factor (HGF) and c-Met: $n = 6$ for each period in each group, transforming growth factor (TGF) β -1: $n = 5$ for each period in each group. ^a $P < 0.05$.

mechanisms of reduction of liver damage are mediated by various effects of HBO, which directly increases the oxygen concentration in the remnant liver and augments the expressions of HGF^[11] and vascular endothelial growth factor (VEGF)^[14].

HGF, known as the scatter factor, has mitogenic, morpho-organogenic, angiogenic, and anti-apoptotic effects. HGF and its receptor c-Met are key factors for liver growth and function. TGF β -1 is an inhibitor of proliferation in hepatocyte cultures^[25]. Data from the present study did not reveal significant differences in mRNA levels of HGF and TGF β -1 with HBO treatment after the partial hepatectomy. However, data did reveal that HBO suppressed c-Met mRNA expression at 15 h after the partial hepatectomy. HGF and c-Met promote liver

regeneration, while TGF β -1 inhibits it^[25]. A balance of these promoter and inhibitor levels is important for liver regeneration^[26]. HBO modulated the expression of c-Met mRNA and might cause an improvement in cholestasis and liver edema.

In conclusion, data from this study suggests that HBO has beneficial effects on the regeneration of biliary ductal cells and postoperative cholestasis after a partial hepatectomy. HBO also improved cell edema of hepatocytes in the regenerating liver. HBO might be useful for improving resectability and for decreasing the frequency of postoperative complications, such as liver failure, after a major hepatectomy.

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COMMENTS

Background

Extended hepatectomy and small graft liver transplantation can result in postoperative cholestasis and liver failure. Postoperative liver failure causes cholestasis; hence, it is important to improve cholestasis in order to reduce morbidity and mortality.

Research frontiers

Hyperbaric oxygenation (HBO) has been used as a therapy in patients with carbon monoxide poisoning, decompression sickness and arterial gas embolism. Effects of HBO which have been reported include upregulation of growth factors, down-regulation of inflammatory cytokines and increased angiogenesis.

Innovations and breakthroughs

The beneficial effects of HBO have been reported in ischemia-reperfusion injuries, acute liver failure after major hepatectomy, hepatic artery thrombosis after liver transplantation and liver regeneration after portal vein embolization. Further to this, the study would suggest that HBO promotes regeneration of biliary cells and improves cholestasis in rats after a partial hepatectomy.

Applications

The study would suggest that HBO has beneficial effects on the regeneration of biliary cells and postoperative cholestasis after a partial hepatectomy. HBO also improved cell edema of hepatocytes in the regenerating liver. HBO might be useful for improving resectability and for decreasing the frequency of postoperative complications, such as liver failure after a major hepatectomy.

Terminology

HBO involves the inspiration of a high concentration of oxygen in a pressure chamber at pressures higher than 1 ATA. During HBO, the partial pressure of oxygen increases, thereby increasing oxygen delivery throughout the body. In general, HBO has been used as a therapy in patients with carbon monoxide poisoning, decompression sickness and arterial gas embolism. Other beneficial effects of HBO in hepatic surgery and liver regeneration have been reported.

Peer review

The authors investigated the effect of HBO administration on bile duct cellular regeneration and cholestasis in a 70% model of hepatectomy. This is an area of high significance as findings may have a potential impact on therapeutic strategies in liver failure and regeneration following major hepatectomy. In limitation, the conclusions are based on results up to 96 h. The regeneration cycle is usually complete by day 8 and it may be appropriate to extend the time interval for the results to be obtained to this stage, before an appropriate conclusion may be reached. It may very well be the optimum results evident are a transient phenomena during the regenerative cycle.

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Limited water infusion decreases pain during minimally sedated colonoscopy

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water group than in the air group (2.5 ± 2.5 vs 3.4 ± 2.8 , mean $\pm$ SD, $P = 0.021$). The cecal intubation time was significantly longer in the water group than in the air group (6.4 ± 3.1 min vs 4.5 ± 2.4 min, $P < 0.001$). More water was infused in the water group (322 ± 80.9 mL vs 26.2 ± 39.4 mL, $P < 0.001$).

CONCLUSION: Limited airless water infusion in the distal colon reduces patients' pain during colonoscopy.

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Key words: Water; Pain; Colonoscopy; Looping; Intubation time

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Abstract

AIM: To investigate a limited water infusion method in colonoscopy.

METHODS: Consecutive patients undergoing minimally sedated colonoscopy were randomized to receive air insufflation ($n = 89$) or water infusion limited to the rectum, sigmoid colon and descending colon ($n = 90$). Completion rates, cecal intubation times, procedure times, need for abdominal compression, turning of patients and levels of discomfort were evaluated.

RESULTS: Completion rates, total procedure times, need for abdominal compression, and turning of patients were similar between groups. Less pain was experienced in the

INTRODUCTION

Colonoscopy is currently the gold standard for the evaluation and treatment of colon disease^[1,2]. The procedure can sometimes be quite painful and the success rate of intubation varies with the skills of the endoscopists^[3]. Various methods have been used to reduce the pain of colonoscopy, including variable stiffness colonoscopy, magnetic endoscope imaging and oil lubrication^[4-6]. Some of these measures require new instruments and their effects are controversial.

Pain during colonoscopy may originate from colon distension caused by air insufflation during the procedure. Adequate air insufflation opens the lumen of the

colon and improves the colonoscopic view. Overinflation, however, elongates the colon, accentuates the angles and results in difficult intubation. In addition, overinflation reduces mucosal blood flow in the laboratory setting and could possibly cause clinically significant ischemia^[7].

Water instillation into the colon can facilitate intubation by straightening the sigmoid colon and decreasing the friction between the scope and the colonic mucosa during colonoscopy^[6]. Recently, an airless water intubation method was proposed in which water, in lieu of air, was used to open the lumen of the colon^[8]. In previous studies, this method was found to decrease the use of sedative medication during sedated colonoscopy and to increase patient willingness to receive unsedated colonoscopy^[8-10]. However, a large amount of water was needed to inflate the colon adequately, and if water was infused manually with a syringe, the procedure time was prolonged^[8]. Otherwise, an automatic infusion pump was needed^[9-11].

In this randomized, controlled study, we simplified the water intubation method by limiting the infusion of water to the rectum and sigmoid colon. We aimed to investigate whether this limited water infusion method could reduce patient discomfort during minimally sedated colonoscopy.

MATERIALS AND METHODS

This prospective study was conducted between May and November 2009 at Buddhist Dalin Tzu Chi General Hospital. Patients undergoing minimally sedated colonoscopy performed by two endoscopists (Hsieh YH and Tseng KC) at our endoscopic suite were included. Patients with the following conditions were excluded: obstructive lesions of the colon, inadequate bowel preparation, allergy to meperidine, massive ascites, past history of partial colectomy, or refusal to provide written informed consent. The study was approved by the Institutional Review Board of the Buddhist Dalin Tzu Chi General Hospital.

Bowel cleansing was accomplished by asking the patients to ingest either 90 mL of sodium phosphate, or 4 bisacodyl tablets (4 × 5 mg) plus 250 mL of magnesium citrate (Purzer Pharmaceutical Co., Taipei, Taiwan) oral solution in two divided doses the night before the procedure.

Buscopan (hyoscine N-butylbromide, 20 mg), if not contraindicated, and 25 mg of meperidine were given intramuscularly immediately before the procedure to produce good colonic relaxation and reduce patient discomfort.

The colonoscopic examinations were performed by using a video colonoscope (CF 240AL, Olympus Optical Co. Ltd., Tokyo, Japan). All procedures were performed with the standard method, including starting with the patient in the left lateral decubitus position, avoiding and reducing loops as much as possible. The variable stiffness function was activated at the discretion of the endoscopist, usually after the sigmoid colon was passed and before manual abdominal pressure was applied. Intubation

of the cecum was defined as successful only if the base of the cecum could be touched with the tip of the colonoscope. Detailed examinations were undertaken during the withdrawal phase.

The patients were allocated into two groups randomly by means of a computer-generated list. In the air group, air insufflation was used throughout the procedure. In the water group, the air pump was turned off initially during the procedure. Instead, tap water in 50 mL syringes was instilled into the rectum and the sigmoid colon through the accessory channel to open the colon lumen. Water was not usually instilled into the descending colon because the infused water already accumulated in this dependent segment. If the tip of the scope came out of the water or could not find its way at the transverse colon, the air pump was then turned on. In addition, if the lumen could not be opened with water in the sigmoid colon after a 5-min attempt, the air pump was turned on.

Demographic data (age, gender, height, weight, and education level), indications for colonoscopy, history of abdominal surgery or hysterectomy, constipation and chronic use of laxatives were obtained before colonoscopy. At the end of the procedure, the following parameters were evaluated and recorded on the patient data sheet: quality of bowel preparation, cecal intubation time, total procedure time, activation of the variable stiffness function, use of abdominal pressure, use of changing position, presence of polyps and reasons for incomplete colonoscopy. Body mass index was calculated as body weight divided by body height squared (kg/m²). The quality of bowel cleansing was classified by the endoscopist as: (1) good: dry colon or only a small amount of clear liquid; (2) fair: large amount of clear liquid fluid or minimal solid stools; (3) poor: significant amount of solid residue; and (4) inadequate: when stool residue precludes complete insertion.

A trained research assistant who was unaware of the randomization status administered a questionnaire to the subjects after the procedure. In the questionnaire, abdominal pain was graded according to 10 levels (VAS scale). The degree of satisfaction was also assessed by a similar scale.

Statistical analysis

A sample size of about 75 patients was chosen to detect a difference of 1 in the patient abdominal pain score during colonoscopy given a standard deviation of 2.2 with a two-tailed α of 0.05 and a β of 0.20. Statistical analysis was performed using SPSS version 12.0 software (SPSS Inc., Chicago, IL). All continuous variables were expressed as mean $\pm$ SD. The Student's *t*-test and an analysis of variance to compare the means of continuous variables were used where appropriate. The χ^2 test, with Yates' correction for continuity, was used for comparison of categorical data, while the Fisher exact test was used when numbers were small. Multivariate logistic regression was performed using the application of assistant-administered abdominal pressure as the end point. A *P*

Table 1 Baseline characteristics of the patients undergoing colonoscopy (mean $\pm$ SD) *n* (%)

	Air group (<i>n</i> = 89)	Water group (<i>n</i> = 90)	<i>P</i> value
Male	51 (57.3)	49 (54.4)	0.764 ²
Age (yr)	58.3 $\pm$ 13.3	57.2 $\pm$ 13.3	0.612 ¹
BMI (kg/m ²)	24.0 $\pm$ 3.5	24.2 $\pm$ 3.5	0.678 ¹
Previous abdomino-pelvic surgery	27 (36.0)	23 (30.7)	0.604 ²
Constipation	34 (38.2)	22 (24.4)	0.054 ²
Inpatients	5 (5.6)	3 (3.3)	0.497 ²
Indications			0.482 ²
Follow-up of polyps	34 (38.2)	29 (32.2)	
Abdominal pain	5 (5.6)	9 (10.0)	
Rectal bleeding	13 (14.6)	11 (12.2)	
Stool occult blood	12 (13.5)	18 (20.0)	
Change of bowel habit	16 (18.0)	18 (20.0)	
Anemia	3 (3.4)	1 (1.1)	
Loss of body weight	3 (3.4)	3 (3.3)	
Other	3 (3.4)	1 (1.1)	
Education level			0.969 ²
Primary school and less	21 (24.1)	21 (24.4)	
High school	38 (42.7)	40 (44.4)	
College and higher	30 (34.5)	29 (33.7)	
Bowel cleansing regimens			0.179 ²
Sodium phosphate	41 (46.1)	51 (56.7)	
Magnesium citrate-bisacodyl	48 (53.9)	39 (43.3)	
Anxiety	3.0 $\pm$ 2.9	3.9 $\pm$ 2.9	0.110 ¹
Antispasmodic agent use	72 (80.9)	75 (83.3)	0.700 ²
Colon preparation			0.276 ²
Good	49 (55.1)	60 (66.7)	
Fair	25 (28.1)	18 (20.2)	
Poor	15 (16.9)	12 (13.3)	

¹Student's *t*-test; ² χ^2 test. BMI: Body mass index.

value of less than 0.05 was considered to be statistically significant.

RESULTS

Between May 2009 and February 2010, 255 patients underwent colonoscopies performed by the two endoscopists at our hospital. Sixty-one patients wished to receive sedation, so they were not invited to participate in the study. Twelve patients were excluded because of prior hemicolectomy (*n* = 6), unwillingness to give written informed consent (*n* = 5), or massive ascites due to carcinomatosis (*n* = 1). The remaining 182 subjects were randomized to the air group (*n* = 90) or the water group (*n* = 92). Three patients were subsequently excluded because of inadequate preparation (one in each group) and severe colitis (one in the water group). The baseline characteristics of the remaining patients in both groups were similar (Table 1).

Incomplete colonoscopy occurred in 1 patient in the air group (1.1%) due to looping. Incomplete colonoscopy also occurred in 1 patient in the water group (1.1%) due to intolerance. However, air insufflation had to be used in 8 patients in the water group before reaching the transverse colon due to poor visibility in 2 patients, and an inability to open the lumen in 6 patients (4 at the sigmoid and 2 at the descending colon). These 8 patients were all subsequently intubated to the cecum.

Table 2 Outcomes of colonoscopy (mean $\pm$ SD) *n* (%)

	Air group (<i>n</i> = 89)	Water group (<i>n</i> = 90)	<i>P</i> value
No. of failed cecal intubations	1 (1.1)	1 (1.1)	1.000 ⁴
Cecal intubation time (min)	4.5 $\pm$ 2.4	6.4 $\pm$ 3.1	< 0.001 ³
Procedure time (min)	13.8 $\pm$ 5.6	14.5 $\pm$ 4.7	0.333 ³
Volume of water used (mL)	26.2 $\pm$ 39.4	322 $\pm$ 80.9	< 0.001 ³
Time of air insufflation during intubation (min)	4.5 $\pm$ 2.4	2.3 $\pm$ 2.6	< 0.001 ³
No. of cases requiring assistant-administered abdominal pressure	51 (57.3)	49 (54.4)	0.764 ⁴
No. of cases requiring change of position	24 (27.0)	17 (18.9)	0.217 ⁴
Patient pain score ¹	3.4 $\pm$ 2.8	2.5 $\pm$ 2.5	0.021 ³
No. of cases without pain	18 (20.2)	32 (35.6)	0.030 ⁴
Patient satisfaction score ²	9.6 $\pm$ 0.8	9.6 $\pm$ 0.7	0.980 ³
No. of cases with polyps	52 (58.4)	44 (48.9)	0.232 ⁴
No. of cases with adenomas	31 (34.8)	32 (35.6)	1.000 ⁴

¹0 = no pain, 10 = worst pain imaginable; ²0 = not satisfied at all, 10 = completely satisfied; ³Student's *t*-test; ⁴ χ^2 test.

The need for abdominal compression, need for changing position and total procedure time were similar between groups (Table 2). The cecal intubation time was shorter in the air group than in the water group (4.5 $\pm$ 2.4 min *vs* 6.4 $\pm$ 3.1 min, *P* < 0.001).

Significantly less water was infused in the air group than in the water group during the procedure (26.2 $\pm$ 39.4 mL *vs* 322 $\pm$ 80.9 mL, *P* < 0.001). The time of air insufflation during the insertion phase was less in the water group than in the air group (4.5 $\pm$ 2.4 min *vs* 2.3 $\pm$ 2.6 min, *P* < 0.001). The mean pain scores, as rated by the patients, were higher in the air group than in the water group (3.4 $\pm$ 2.8 *vs* 2.5 $\pm$ 2.5, *P* = 0.021). Also, more patients in the water group had no pain at all compared to patients in the air group (35.6% *vs* 20.2%, *P* = 0.030). Overall satisfaction with the procedure was similar between groups (9.6 $\pm$ 0.8 *vs* 9.6 $\pm$ 0.7, *P* > 0.05) (Table 2).

Polyps were detected in 52 (58.4%) patients in the air group. Thirty-one (34.8%) of these polyps were adenoma, 5 (5.6%) were tubulovillous adenoma and 3 (3.4%) were carcinoma. Polyps were detected in 44 (48.9%) patients in the water group. Thirty-two (35.6%) polyps were adenoma, 4 (4.4%) were tubulovillous adenoma and 1 (1.1%) was carcinoma (Table 2).

DISCUSSION

The results of this study show that water infusion rather than air insufflation at the rectum and sigmoid is associated with less pain during minimally sedated colonoscopy. In experienced hands, about 35.6% of the patients felt no pain at all. The water was infused manually with a syringe without additional instruments, making this method readily available. Although the limited water infusion group had a longer intubation time than the air insufflation group, the difference was small (less than 2 min). To the best of our knowledge, this is the first study to compare this new water infusion method with traditional air insufflation.

Water infusion to facilitate colonoscopy has been reported by several authors in the past; however it has not been applied frequently. Most authors used water infusion in conjunction with air insufflation. Falchuk *et al.*^[12] found it helpful to infuse up to 300 mL of water into the sigmoid colon while intubating patients with severe diverticulosis. Baumann compared water infusion (200 mL) with traditional air insufflation during colonoscopic examination^[13]. He found that passing through the left colon was faster with the water method than with the air method. Brocchi *et al.*^[6] compared warm water (300 mL) infusion with seed oil and traditional air insufflation during colonoscopic examination. They found that water infusion was associated with a higher cecal intubation rate, shorter intubation time and less pain than in the control group.

Recently, Leung *et al.*^[9] employed a novel method with infusion of a large amount of water during the insertion phase. In their pilot study, airless water intubation permitted 52% of patients to complete the procedure without sedation. A mean volume of more than 1 L of water was infused in aliquots of 30 to 60 mL. The major drawback of this technique was the long intubation time of up to 22.6 min^[9]. In a subsequent randomized, controlled study comparing water intubation with air insufflation, the water was infused intermittently with a peristaltic pump with a blunt needle adaptor through the biopsy channel^[10]. The cecal intubation times were comparable between the two groups. The endoscopists found that the increments of medications and the maximum pain scores were significantly lower with the water method^[10].

Our study showed that a lower mean pain score was experienced in the water group (2.5 ± 2.5) than in the air group (3.4 ± 2.8 , $P = 0.021$). A previous study has shown that patient discomfort occurs when the colonoscope tip reaches the sigmoid colon^[14]. Loop formation of the colonoscope occurring mostly in the sigmoid colon is the major cause of pain^[15]. We infused water instead of air in the rectum and sigmoid colon in our study. By eliminating air in the sigmoid colon, we reduced the loop formation and caused less pain in these patients. The weight of the water might also be helpful in reducing looping over the sigmoid colon.

In the present study, we did not use air insufflation until the scope reached the transverse colon. The infused water accumulated at the descending colon due to gravity when patients were in the left lateral decubitus position. When the scope reached the transverse colon the air was switched on, otherwise much more water would have been needed to open the more proximal colon, which was at non-dependent areas. By limiting the use of water infusion to the distal colon, we could achieve intubation with little water (around 300 mL). This amount of water could easily be infused with a syringe instead of a peristaltic pump.

The intubation time was longer in the water group than in the air group (6.4 ± 3.1 min *vs* 4.5 ± 2.4 min). However, air had to be turned on prematurely in 8 (8.9%) patients in the water group. There are several reasons for

the longer time in the water group. Firstly, it took time to infuse water into the biopsy channel repeatedly with a syringe. Secondly, it was difficult to open a collapsed segment or acute angle at a non-dependent portion without enough water. Thirdly, the view was less clear and the lumen was more difficult to find under water than with air insufflation, especially when the preparation was less than optimal. With a limited amount of water infused, our method had little cleansing effect on colon contents^[8].

The polyp detection rates were similar in our study groups. After opening the lumen adequately with air insufflation and aspirating the residual stools and fluid, we inspected the mucosa closely when we withdrew the scope in both groups, so the infused water did not impair our ability to detect polyps. When the bowel preparation was adequate, we had no difficulty finding a polyp under water during insertion.

In this study, we infused water at room temperature. Some previous studies used warm water^[6,10,16], but others have used water at room temperature^[13,17]. Church *et al.*^[16] showed that warm water minimized colon spasms and decreased patient discomfort. Most of our patients had received intravenous buscopan before colonoscopy, so we did not encounter any colon spasms in this study.

This study has several limitations. The procedures were performed by two experienced endoscopists, since the procedure conditions cannot appropriately be handled by less experienced doctors. The endoscopists were not blinded to the methods, but the patients were blinded. The use of the water method only reduced a small proportion (about 26%) of the pain scores; however the procedure was simple and cost-effective. We did not compare the syringe infusion method with a peristaltic pump method, although this does warrant further studies.

In conclusion, compared with traditional air insufflation, limited infusion of water at the distal colon resulted in less pain in patients undergoing minimally sedated colonoscopy, although a longer intubation time was required.

COMMENTS

Background

Colonoscopy can sometimes be quite painful. Pain during colonoscopy may originate from colon distension caused by air insufflation. Water instillation into the colon can facilitate intubation by straightening the sigmoid colon and decreasing the friction between the scope and the colonic mucosa. The weight of water also helps prevent loop formation.

Research frontiers

Recently, an airless water intubation method was proposed in which water, in lieu of air, was used to open the lumen of the colon. In previous studies, this method was found to decrease the use of sedative medication during sedated colonoscopy. However, a large amount of water was needed to inflate the colon adequately, which might increase procedure time or require an additional peristaltic pump. In this randomized, controlled study, the authors simplified the water intubation method by limiting the infusion of water to the rectum and sigmoid colon.

Innovations and breakthroughs

In the limited water group in the present study, the air pump was turned off initially during the procedure. Tap water in 50 mL syringes was instilled into the rectum and the sigmoid colon through the accessory channel to open the colon lumen. Water was not usually instilled in the descending colon because the

infused water already accumulated in this dependent segment. The air pump was opened when the colonoscope reached the transverse colon. No additional instrument was needed with this method and only about 300 mL of water was used to complete the colonoscopy.

Applications

This article shows that limited airless water infusion in the distal colon reduces patients' pain during colonoscopy. Further research should be done by less experienced endoscopists. In addition, head-to-head comparison of the limited water infusion method with total water infusion is needed.

Peer review

Hsieh *et al* present the results of the largest randomized trial yet performed to assess the value of water instillation to facilitate colonoscopy, here in minimally sedated patients. In general the paper is well written, the methodology is good and the results will generate widespread interest amongst endoscopists.

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Mechanism and dose-effect of Ginkgolide B on severe acute pancreatitis of rats

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Abstract

AIM: To determine the optimal dosage and mechanism of Ginkgolide B (BN52021) on severe acute pancreatitis (SAP) of rats.

METHODS: Seventy male Wistar rats were randomly divided into seven groups (10 for each group). Sham-operation group (SO), SAP model group (SAP), dimethyl sulfoxide (DMSO) contrast group (DMSO), and groups treated with 2.5 mg/kg BN52021 (BN1), 5 mg/kg BN52021 (BN2), 10 mg/kg BN52021 (BN3), and 20 µg/kg Sandostatin (SS). The SAP model was established in Wistar rats by injecting 5% sodium taurocholate retrogradely into the common bilio-pancreatic duct. The rats of SO, DMSO and BN52021 were injected with 0.9% NaCl, 0.5% DMSO and BN52021 through femoral vein 15

min after the operation. The SS group was injected with Sandostatin subcutaneously. All rats were anaesthetized at 6 h after operation, and venous blood was collected to determine the levels of serum amylase and phospholipase A2 (PLA₂), and pancreas tissue was harvested and stained.

RESULTS: There was no significant difference between the SAP and DMSO groups in serum amylase level, PLA₂, ascites and pathologic score, but significant difference was found in SAP/DMSO groups compared with those in SO group ($P < 0.05$) and the levels of serum amylase, PLA₂, ascites, and pathologic score were lower in the BN1, BN2, BN3 and SS groups than in the SAP and DMSO groups ($P < 0.05$). However, among BN1, BN2, BN3 and SS groups, BN2 had the best effect in decreasing the levels of serum amylase and PLA₂ ($P < 0.05$). Expression of platelet activating factor (PAF) receptor (PAFR) mRNA and protein showed no significant difference between the SAP and DMSO groups, or among BN1, BN2, BN3 and SS groups, but there was remarkable difference between SAP/DMSO group and SO group ($P < 0.05$), and expression of PAFR mRNA and protein was higher in the BN1, BN2, BN3 and SS groups than in the SAP and DMSO groups ($P < 0.05$). PAFR expression was observed in the nucleus and cytoplasm of pancreatic islet cells in Wistar rats by immunohistochemistry.

CONCLUSION: By iv injection, 5 mg/kg of BN52021 is the optimal dosage for SAP rats. BN52021 may inhibit the interaction/binding of PAF with PAFR.

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Key words: Pancreatitis; Ginkgolide B; Dose-effect; Phospholipase A2; Platelet activating factor receptor

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INTRODUCTION

Platelet activating factor (PAF) is a biologically active phospholipid mediator, playing its role by binding to PAF receptor (PAFR) which is a unique G-protein-coupled seven transmembrane receptor, and it activates multiple intracellular signaling pathways^[1]. Flickinger *et al.*^[2] revealed specific localization of PAFR in the pancreatic vascular endothelium, but not in other pancreatic cell types. Recent studies demonstrated that PAF played an important role in the pathological progress of severe acute pancreatitis (SAP)^[3].

Ginkgolide B (code: BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J), which are present in the whole extract of Ginkgo biloba leaves. It has been found that BN52021 possesses activity as antagonists of the action of PAF. It can inhibit PAF-induced cascade effect in inflammatory reactions. For example, it can reduce the portal vein pressure of liver cirrhosis, exhibit an anti-shock effect, and exert protective functions on experimental asthma^[4,5].

SAP is dangerous to life. It can cause multiple organ system failure (MOSF) and if the patients do not receive effective treatment in time, they may die. But up till now, there has been no effective drug for this disease. Recent studies have shown that BN52021 has best effect on experimental SAP^[6,7], but there is no report on its optimal dosage, and its mechanism has not been fully understood. Especially as an extract of Ginkgo biloba, it is hypotoxic, and can be used in clinical treatment of SAP. We investigated the optimal dosage of BN52021 and its mechanism on SAP.

MATERIALS AND METHODS

Main reagents and apparatus

BN52021 and sodium taurocholate were purchased from Sigma (St. Louis, MO, USA), amylase kit from Beijing Kemei Reagent Co. (Beijing, China), trizol and diethyl pyrocarbonate (DEPC) from Invitrogen (Carlsbad, CA, USA), RT kit from MBI Fermentas (Ontario, Canada), DNTPs and RNA enzyme inhibitor from TaKaRa Dalian Co., Ltd. (Dalian, China), DNA Tag enzyme from Promega (Madison, WI, USA), primary antibody of PAF-R rabbit-anti-rat serum and the enhanced chemiluminescence system from Santa Cruz Biotechnology (Santa Cruz, CA, USA), secondary antibody of sheep-anti-rabbit from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China), β -actin from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China), DYY-12 electrophoresis system and electric trans-

blot SD from Beijing Liuyi Instrument Factory (Beijing, China), Type-2720 polymerase chain reaction (PCR) apparatus from ABI (Foster, CA, USA), and gel scanning and imaging system and vertical electrophoresis system from Bio-Rad (Hercules, CA, USA).

Primer sequences

PAF-R and β -actin primer series are provided by Invitrogen (Carlsbad, CA, USA): PAF-R: forward primer (F): 5'-CCGCTGTGGATTGTCTATTA-3', reverse primer (R): 5'-AGGAGG TGATGAAGATGTGG-3' (377 bp)^[8]; β -actin: F: 5'-TCC TAGCACCATGAAGATC-3', R: 5'-AAACGCAGCTCAGTAACAG-3' (190 bp)^[9].

Experimental animal grouping and model preparation

Seventy Wistar male rats (Laboratory Animal Center of PLA, Academy of Military Medical Sciences, Beijing, China), aged 6-8 wk, and weighing 200-220 g (Grade II, Certificate SCXK 2002-001) are used for this study. All rats were maintained in an environment of controlled temperature (22-25°C), humidity (55%-58%), and lighting (12 h light/12 h dark), with free access to tap water and regular chow diet. Rats were divided randomly into 7 groups: Sham-operation group (SO group, $n = 10$), SAP model group (SAP group, $n = 10$), DMSO contrast group (DMSO group, $n = 10$), and treatment groups of 2.5 mg/kg BN52021 (BN1 group, $n = 10$), 5.0 mg/kg BN52021 (BN2 group, $n = 10$), 10.0 mg/kg BN52021 (BN3 group, $n = 10$), and 20.0 μ g/kg Sandostatin (SS group, $n = 10$)^[10]. SAP model was established according to Aho *et al.*^[10]. All rats were weighed, marked and fasted for 24 h before operation. The rats were anesthetized by abdominal injection of 0.4% pentobarbital sodium (40 mg/kg), and fixed in dorsal decubitus. A 2-cm cut was made at the center of the upper belly, entering the abdominal cavity to look for rat's duodenum and pancreaticobiliary duct. The hepatic end of the pancreaticobiliary duct was clipped with a non-invasive vascular clip, and pancreaticobiliary duct retrograde centesis was performed with an obtuse needle through duodenum seromuscular layer. Then 5% sodium taurocholate (0.1 mL/100 g) was injected in the retrograde direction of pancreaticobiliary duct with a micro-syringe at an injection rate of 0.20 mL/min. After injection, the port of pancreaticobiliary duct entering duodenum was clipped with a non-invasive vascular clip and observed for 10 min. When no active bleeding was confirmed in the abdominal cavity, the abdomen was closed in two layers and the incision was covered with sterile gauze. Same treatment was given to the SO group but without injections into the duct. Fifteen min after the model was completed, the rats in SO and SAP groups were injected with physiological saline (0.9% NaCl) through femoral vein at a dose of 5ml/kg; the rats of DMSO group were injected with DMSO (0.5% DMSO) at a dose of 5ml/kg; the BN1, BN2, and BN3 groups were rapidly injected with BN52021 at doses of 2.5, 5.0 and 10.0 mg/kg, dissolved with 0.5% DMSO; and SS group was injected with Sandostatin subcutaneously at a dose of 20.0 μ g/kg^[11].

Collection and storage of samples

Rats in each group were anaesthetized at 6 h after first injection of Sodium taurocholate, because the $T_{1/2}$ of BN52021 is 5.23 h^[12], venous blood was collected from right atrium and was centrifuged for 10 min at 3000 g/min after a 10-min water bath at 37°C. The supernatant was placed into a sterilized EP tube, and stored in refrigerator at -20°C for serum amylase determination. Portions of pancreatic tissues were placed in liquid nitrogen overnight and frozen in refrigerator at -80°C, meanwhile, another portion of pancreatic tissues was fixed with 40 g/L neutral buffer formaldehyde, embedded in paraffin wax, cut into slices and stained with HE for pathological observation and scoring. Pneumonic tissues were fixed with 40 g/L neutral buffer formaldehyde, embedded in paraffin wax, and cut into slices for immunohistochemical studies.

Determination of serum amylase, PLA2 and pathological observation, scoring of pancreatic tissues

Serum amylase and PLA₂ were detected with fully automatic biochemical apparatus, amylase and PLA₂ kits. Pancreatic tissue samples were observed pathologically and scored according to the reference^[13].

Determination of PAFR mRNA expression by reverse transcription PCR

After total RNA from pancreatic tissue in each group was extracted, its integrality was checked with agarose electrophoresis, and its concentration and purity were determined with an UV spectrophotometer, and the concentration was calculated. RNA 5 µg and Oligo DT₁₅ 1.25 µg were placed into a water bath at 70°C for 5 min, rapidly put into ice for 5 min, and centrifuged just for 15 s. 5 × M-MLV reverse transcription buffer 5 mL, DNTPS 0.05 µmol, RNA enzyme inhibitor 40 U, M-MLV and reverse transcriptase 1 µL were added, and diluted to 25 µL with deionized water treated by DEPC. The whole mixture was maintained at 42°C for 60 min. Reverse transcription was conducted and reverse transcriptase was deactivated at 95°C for 5 min. The mixture was maintained at 4°C for 5 min and preserved at -20°C. Fifty microliters PCR reaction system consists of 3 µL reverse transcription product, 0.01 µmol DNTPS, 5 µL 10 × PCR buffer, 0.075 mol MgCl₂ (Magnesium Chloride), 50 pmol of each PAF-R specific sense strand and anti-sense strand, and 50 pmol of each β-actin sense strand and anti-sense strand. Reaction conditions were as follows: pre-denaturizing at 94°C for 3 min, denaturizing at 94°C for 45 s, annealing at 58°C for 45 s, and extending at 72°C for 45 s, 38 cycles in total; extending at 72°C for 7 min, and preserving at 4°C. The PCR product was included in the PAF-R gene sequence of rat spleen (gi:470384), which was performed by Beijing Boya Biotechnology Co., Ltd. The PCR product was analyzed by 2% agarose gel electrophoresis, ethidium bromide staining, and a gel imaging system scanning. Gray level of PCR product was treated by PAF-R and β-actin was analyzed using the Quantity-One Software, and the change of PAF-R mRNA expression was evaluated semi-quantitatively.

Determination of PAFR protein expression by immunohistochemistry

Paraffin slices of pancreas (5 µm) were made, mounted on slides and treated with paraformaldehyde-lysine-periodate solution. The slides were immersed in 100% dimethylbenzene for 15 min, 100% ethanol for 10 min, 95% ethanol for 5 min, 80% ethanol for 5 min, 70% ethanol for 5 min, and washed with phosphate-buffered saline (PBS). The slides were immersed in citrate solution for 15 min in microwave at high temperature, cooled at room temperature, washed for 3 times with PBS, incubated with the rabbit polyclonal antibody against a recombinant protein corresponding to amino acids at the amino terminus of PAFR (1:100) for 18 h at 4°C, then incubated with goat anti-rabbit IgG-HRP polymer (1:500) for 0.5 h at 4°C, washed with PBS after incubation, and then incubated with DABH₂O₂ solution for color development, and washed with lotic water. The slices were counterstained with hematoxylin and mounted with coverslips. The slices were observed and analyzed, and the quantity of PAFR protein expression was determined according to the density of positive cells. Some frozen slices of pancreatic tissues and paraffin slices of lung tissues were made to observe the location of PAFR in pancreatic and lung tissues by immunohistochemistry.

Statistical analysis

The experiments were repeated for 3 times and their average values served as the final values; all values were expressed as mean ± SD. Data were processed with SPSS 11.5 Statistical Software, and LSD and Dunnett's post-hoc method were used for comparison of ANOVA. Statistical significance was established at $P < 0.05$.

RESULTS

Serum level of amylase and PLA₂, quantity of ascites and pathological scores

There were no significant differences in the serum levels of amylase and PLA₂, quantity of ascites and pathological scores between SAP and DMSO groups. It was higher in SAP/DMSO group than in SO group ($P < 0.05$), and lower in BN1, BN2, BN3 and SS group than in SAP/DMSO group ($P < 0.05$). However, BN2 had the best effect in decreasing the serum level of amylase and PLA₂ among BN1, BN2, BN3 and SS groups ($P < 0.05$) (Table 1, Figure 1).

Expression of PAFR mRNA and its proteins in pancreatic tissues

There was no significant difference in expressions of PAFR mRNA and PAFR protein between SAP and DMSO groups, neither among BN1, BN2, BN3 and SS groups. Expression of PAFR mRNA and its proteins in pancreatic tissues was lower in SAP/DMSO group than in SO group ($P < 0.05$), and higher in BN1, BN2, BN3 and SS groups than in SAP/DMSO group ($P < 0.05$) (Table 2, Figures 2 and 3).

Location of PAFR in pancreatic and lung tissues

PAFR was located in pancreatic microvascular endothelial

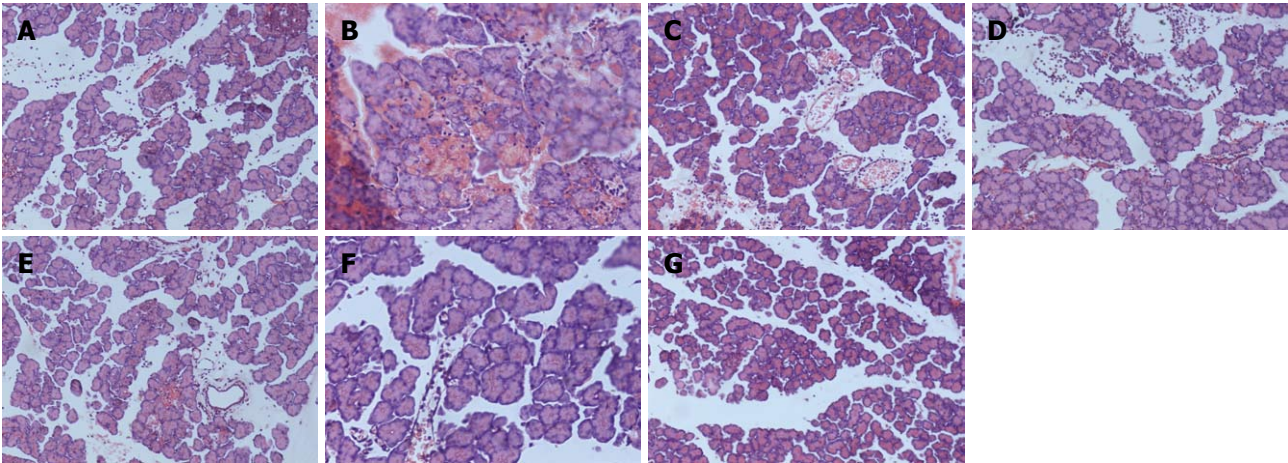


Figure 1 Pathological change of pancreatic tissues (HE staining, × 200). A: Sham-operation group; B: Severe acute pancreatitis group; C: DMSO group; D: 2.5 mg/kg BN52021 group; E: 5.0 mg/kg BN52021 group; F: 10 mg/kg BN52021 group; G: 20 μg/kg Sandostatin group.

Table 1 Serum levels of amylase and PLA ₂ , quantity of ascites and pathological scores (n = 10) (mean ± SD)				
Group	Amylase (U/L)	PLA ₂ (μmol/min per milliliter)	Ascites (mL)	Pathological scores
SO	1923 ± 527 ^{a,c}	0.0434 ± 0.019 ^{a,c}	0.0 ^{a,c}	0 ^{a,c}
SAP	6448 ± 240 ^c	0.2990 ± 0.0225 ^c	5.4 ± 1.3 ^c	9.15 ± 0.55 ^c
DMSO	6070 ± 464 ^c	0.2728 ± 0.4461 ^c	5.0 ± 2.0 ^c	9.10 ± 0.65 ^c
BN1	4744 ± 1031 ^{a,c}	0.2320 ± 0.0890 ^{a,c}	2.4 ± 0.9 ^a	6.65 ± 0.42 ^a
BN2	3452 ± 548 ^a	0.1049 ± 0.0528 ^a	1.6 ± 0.5 ^a	5.55 ± 0.36 ^a
BN3	4777 ± 168 ^{a,c}	0.1069 ± 0.0440 ^a	2.6 ± 0.9 ^a	5.90 ± 0.19 ^a
SS	4646 ± 675 ^{a,c}	0.1632 ± 0.0523 ^{a,c}	2.4 ± 0.5 ^a	5.70 ± 0.30 ^a

PLA₂: Phospholipase A₂; SO: Sham-operation; SAP: Severe acute pancreatitis; BN1: 2.5 mg/kg BN52021; BN2: 5.0 mg/kg BN52021; BN3: 10 mg/kg BN52021; SS: 20 μg/kg Sandostatin. ^a*P* < 0.05 vs SAP group; ^c*P* < 0.05 vs BN2 group.

Table 2 Expression of platelet activating factor receptor in pancreatic tissues (n = 10) (mean ± SD)		
Group	Ratio of PAFR/β-actin mRNA	IOD of PAFR
SO	0.46 ± 0.10 ^a	5394.6 ± 1739.4 ^a
SAP	0.38 ± 0.08 ^c	1881.2 ± 376.7 ^c
DMSO	0.39 ± 0.09 ^c	2105.4 ± 345.2 ^c
BN1	0.54 ± 0.14 ^a	19966.7 ± 6488.7 ^a
BN2	0.60 ± 0.09 ^a	20935.6 ± 6551.9 ^a
BN3	0.60 ± 0.09 ^a	2169.7 ± 6872.5 ^a
SS	0.59 ± 0.08 ^a	20897.5 ± 666.2 ^a

PAFR: Platelet activating factor receptor; IOD: Integrated optical density; SO: Sham-operation; SAP: Severe acute pancreatitis; BN1: 2.5 mg/kg BN52021; BN2: 5.0 mg/kg BN52021; BN3: 10 mg/kg BN52021; SS: 20 μg/kg Sandostatin. ^a*P* < 0.05 vs SAP group, ^c*P* < 0.05 vs BN2 group.

cells and bronchia epithelial cells. It was first found in the nuclei of pancreatic islet cells and cytoplasm of Wistar rats (Figure 4).

DISCUSSION

Amylase, PLA₂, score of pathologic changes of pancreas and quantity of ascites were reduced after administration of BN52021 at 2.510 mg/kg, and the pancreas PAFR mRNA or protein was protected. PAFR expression was found in the pancreas' islet, which has not been reported by others

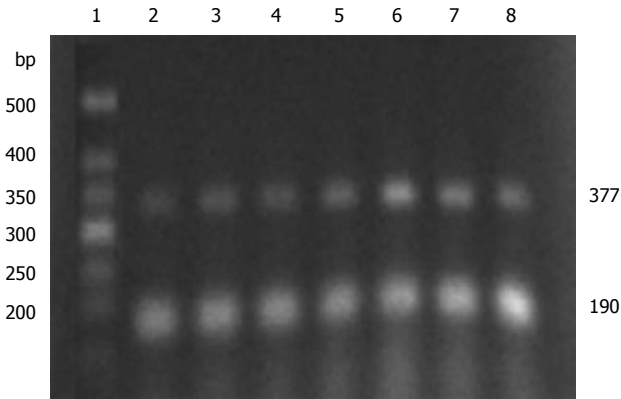


Figure 2 Expression of platelet activating factor receptor mRNA in pancreatic tissues by reverse transcription polymerase chain reaction. 1: Marker; 2: Sham-operation group; 3: Severe acute pancreatitis group; 4: DMSO group; 5: 2.5 mg/kg BN52021 group; 6: 5.0 mg/kg BN52021 group; 7: 10 mg/kg BN52021 group; 8: 20 μg/kg Sandostatin group.

up till now. The best effect was shown at 5 mg/kg among BN groups. BN52021 at 5 and 10 mg/kg showed equivalent effects on SAP, and 10 mg/kg was less effective such as in serum amylase. This may be caused by the dose range between 10 and 5 mg/kg.

PLA₂ is pivotal in the pathogenesis of SAP. There are two types of PLA₂: cytosolic phospholipase A₂ (cPLA₂) and secretory PLA₂ (sPLA₂). There is a cross-talk between

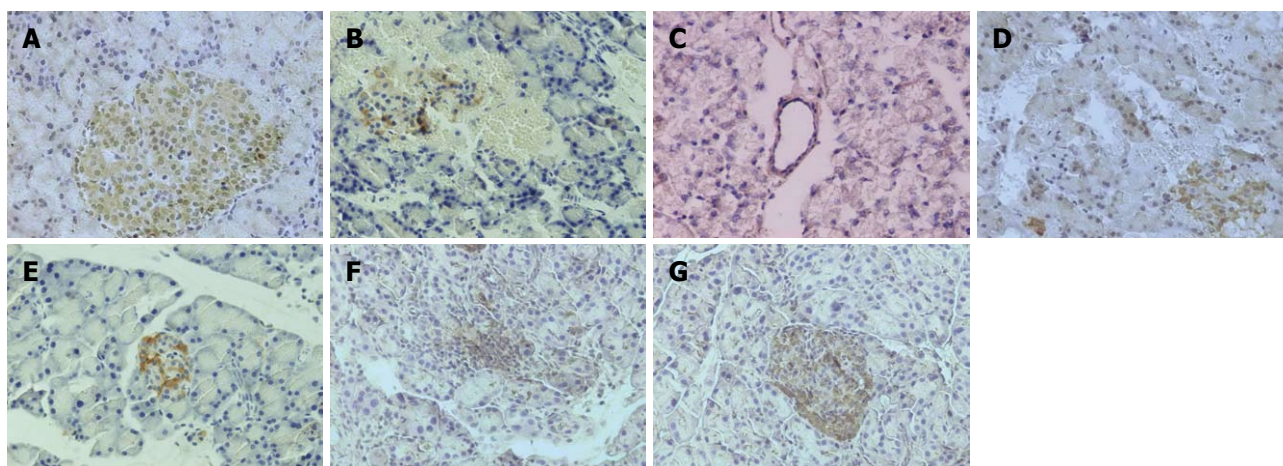


Figure 3 Expression of platelet activating factor receptor in pancreatic tissues by immunohistochemistry (HE staining, $\times 200$). A: Sham-operation group; B: Severe acute pancreatitis group; C: DMSO group; D: 2.5 mg/kg BN52021 group; E: 5.0 mg/kg BN52021 group; F: 10 mg/kg BN52021 group; G: 20 μ g/kg Sandostatin group.

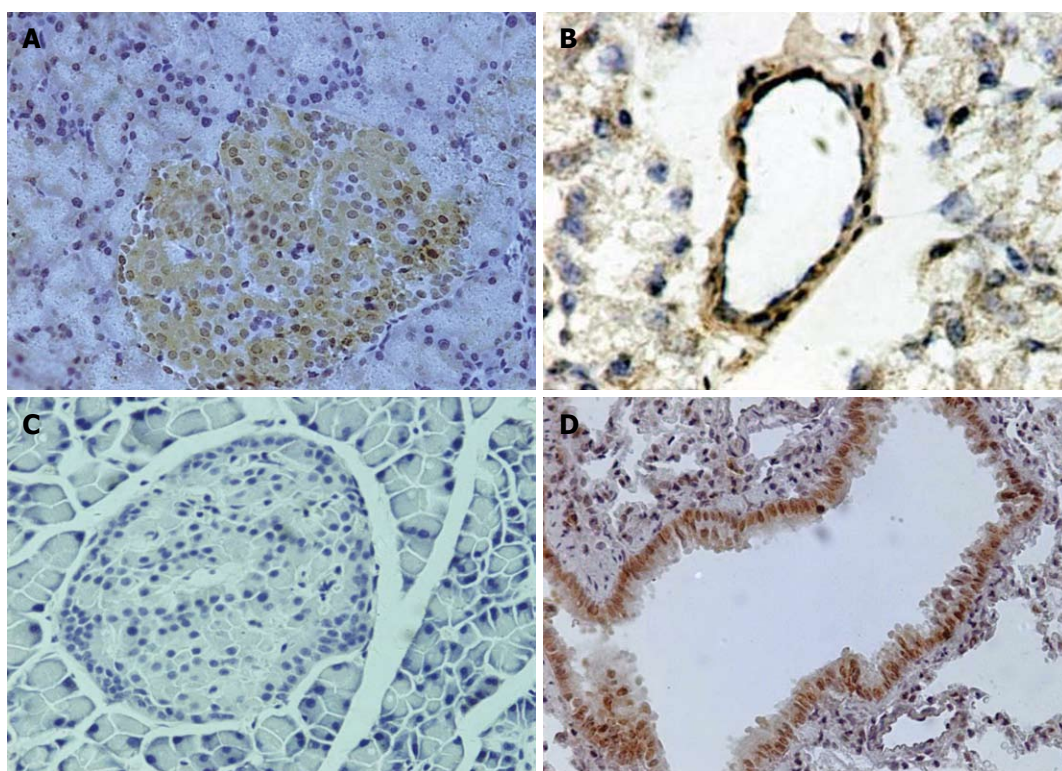


Figure 4 Location of platelet activating factor receptor in pancreatic and lung tissues in sham-operation group shown by immunohistochemistry (HE staining, $\times 200$). A: Paraffin slice of pancreatic tissues; B: Frozen slice of pancreatic tissues; C: Negative control of pancreatic tissues; D: Positive control of lung tissues.

sPLA₂ and cPLA₂. sPLA₂ activates a signaling cascade, including activation of cPLA₂, arachidonic acid release, PAF production, and induction of COX-2^[14]. PLA₂ can decompose the membrane to release PAF. It is known that PAF is an initiative factor in the pathogenesis of SAP, and the cascade exacerbates the course of pancreatitis by activating the PLA₂ in membrane. BN52021 antagonizes PAF by restraining the activation of PLA₂ and this is the first report about the relation between

BN52021 and PLA₂ in SAP.

Quantity of ascites showed the severity of pathological changes of pancreas. When pancreas is involved in inflammation, hemorrhage and putrescence, it would irritate peritoneum and peritoneal permeability was increased, resulting in the release of ascites. PLA₂ was closely correlated with the release of ascites^[15]. The results indicated that BN52021 can reduce the PLA₂ in plasma and decrease the quantity of ascites, and that BN52021 may interact defi-

nately on other inflammatory factors to reduce peritoneal permeability.

The level or regulation of PAFR mRNA and PAFR protein has not been clearly studied. In our experiments, expression of PAFR in pancreas was up-regulated in the initial 3 h in SAP, but down-regulated after 6 h^[16]. The reason for this may be in the first 3 h, pancreas could release more inflammatory factors to induce the up-regulation of PAFR, such as tumor necrosis factor α ^[17] and interferon- β , but cellular membrane was destroyed completely along with severe damages occurring in the pancreas after 6 h. In addition, protein kinase C excess activation was reported to decrease expression of PAFR^[18], followed by a down-regulation of mRNA. BN52021 protected the membrane so that it maintained the quantity of PAFR. There was significant difference between SAP and BN52021 groups. The ratio of IOD of PAFR to β -actin was gradually improved in 2.5 and 10 mg/kg BN52021 groups, although there was no significant difference among BN52021 groups, and remarkable difference was found among SO group, 5 mg/kg SAP group and 5 mg/kg BN group.

It was firstly reported that PAFR was located in pancreas' islet, and it is important to study the mechanism of pancreas inflammation. BN52021 exerted its protective effects in patients with diabetes and pancreatitis, but the active mechanism is not clear. When inflammation in pancreas happened, PAFR was up-regulated, and the cells released more PAF, which damaged the pancreas. As BN52021 competitively inhibited the combination of PAF and PAFR and released less PAF, the pancreas was protected. It meant that BN52021 achieved its effects on SAP not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas.

In conclusion, 5 mg/kg BN52021 is the optimal dose in treating SAP rats, which can reduce the amylase and PLA₂ in serum and the quantity of ascites. It prevents the pancreas from severe damage induced by sodium taurocholate. Compared with Sandostatin, a classical drug for SAP, BN52021 exerts the best effects in treating SAP rats. This paper also showed that BN52021 achieves therapeutic effects in SAP rats not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas. Because of its hypotoxicity as an extract of *Ginkgo biloba*, it will be of significance in clinical application. More experiments are needed for the extensive application of BN52021 in clinic.

COMMENTS

Background

Platelet activating factor (PAF) is a family of phosphorylcholine esters with diverse potent physiological effects. It is closely associated with the cell membrane and is found in a variety of cells. Numerous cell types and tissues have been shown to synthesize and release PAF upon stimulation and at the same time to exhibit biological responses to it. Flickinger *et al* found the specific localization of PAF receptor (PAFR) in the pancreatic vascular endothelium, but not in other pancreatic cell types.

Research frontiers

Recent studies demonstrated that PAF played an important role in the pathological progress of severe acute pancreatitis (SAP). It has been found that BN52021 possesses activity as antagonists of the action of PAF. However, there is no report on its optimal dosage. Although BN52021 has shown best effects on treating SAP, its mechanism has not been elucidated in details. This paper investigated the optimal dosage of BN52021 and its mechanism on SAP.

Innovations and breakthroughs

Recent reports have highlighted the importance of PAF in SAP and have shown that BN52021 has best effect on experimental SAP. This is the first study to report the optimal dosage of BN52021. The authors discovered that there is PAFR expression in the pancreas' islet, which has not been reported by others up to now. The *in vitro* studies suggest that this protein may be the cause of the repression in E-cadherin observed in this cancer.

Applications

By understanding how PAF is induced and by blocking its expression, this study may represent a future strategy for SAP treatment with BN52021.

Terminology

PAF is a biologically active phospholipid mediator, playing its role by binding to platelet activating factor receptor (PAFR) which is a unique G-protein-coupled seven transmembrane receptor, and it activates multiple intracellular signaling pathways, including the nuclear PAFR. Ginkgolide B (code:BN52021) is one of the four Ginkgolide constituents (Ginkgolide A, B, C and J), which are present in the whole extract of *Ginkgo biloba* leaves.

Peer review

The authors examined the level of serum amylase and PLA₂, quantity of ascites, and pathological scores, the expression of PAFR mRNA and its proteins in pancreatic tissues and location of PAFR in pancreatic and lung tissues. It revealed that PAFR expressed in the pancreas' islet and the best effect on SAP was shown in the 5 mg/kg group. This paper also showed that BN52021 achieves the therapeutic action on SAP rats not only by reducing the quantity of PAFR, but also by inhibiting the combination of PAF and PAFR and maintaining the integrity of the cells of pancreas. The results are interesting and may represent a molecular mechanism of BN52021 on SAP.

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Specific HLA-DQB1 alleles associated with risk for development of hepatocellular carcinoma: A meta-analysis

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RESULTS: Among the five family alleles, two (DQB1*02 and DQB1*03) were found to be significantly associated with the risk of HCC. The combined OR for the association of DQB1*02 and DQB1*03 allele with the risk for HCC was 1.78 (95% CI: 1.05-3.03, $P = 0.03$) and 0.65 (95% CI: 0.48-0.89, $P = 0.007$), respectively. Among the 13 specific alleles, two (DQB1*0502 and DQB1*0602) were significantly associated with risk of HCC. The combined OR for the association of DQB1*0502 and DQB1*0602 allele with the risk for HCC was 1.82 (95% CI: 1.14-2.92, $P = 0.01$) and 0.58 (95% CI: 0.36-0.95, $P = 0.03$), respectively. No significant association was established for other HLA-DQB1 family alleles and specific alleles.

CONCLUSION: Our results support the hypothesis that specific HLA-DQB1 allele families and alleles might influence the susceptibility or resistance to HCC, although it needs further investigations.

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Key words: Hepatocellular carcinoma; Human leukocyte antigen-DQB1 alleles; Meta-analysis

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Xin YN, Lin ZH, Jiang XJ, Zhan SH, Dong QJ, Wang Q, Xuan SY. Specific HLA-DQB1 alleles associated with risk for development of hepatocellular carcinoma: A meta-analysis. *World J Gastroenterol* 2011; 17(17): 2248-2254 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i17/2248.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i17.2248>

Abstract

AIM: To evaluate the association of human leukocyte antigen (HLA)-DQB1 alleles with hepatocellular carcinoma (HCC) through meta-analysis of published data.

METHODS: Case-control studies on HLA-DQB1 allele association with HCC published up to January 2010 were included in the analyses. The odds ratios (ORs) of HLA-DQB1 allele distributions in HCC patients were analyzed and compared with healthy controls. The meta-analysis software REVMAN 5.0 was applied for investigating heterogeneity among individual studies and for summarizing all the studies. A meta-analysis was performed using fixed-effect or random-effect methods, depending on the absence or presence of significant heterogeneity. Seven case-control studies containing 398 cases and 594 controls were included in the final analysis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most com-

mon cause of cancer-related deaths worldwide and about 600 000 patients died from the disease annually^[1]. Nearly 78% of the 600 000 cases are from Asian countries^[2]. China alone accounts for more than 50% of the world's cases^[3]. The development of HCC is linked to the interaction of the genetic, immunologic, environmental, dietary, and lifestyle factors. Its incidence and distribution vary widely among ethnic groups, sex, and geographic regions. HBV and HCV infection, liver cirrhosis, male gender, and old age are important risk factors of HCC. The clustering of HCC within families raises the possibility that genetic factors are also involved in the susceptibility to HCC.

The major histocompatibility complex (MHC) plays a key role in anti-virus and tumor defense. Human leukocyte antigens (HLA) function in the regulation of immune response to foreign antigens and discrimination of self from non-self antigens. They are encoded by a series of closely linked genetic loci found on chromosome 6^[4,5]. HLA polymorphism is implicated in conferring genetic susceptibility to a large number of immune-mediated diseases, including some cancers. Given the pivotal role of HLA molecules in the immune system, several studies have investigated the association between specific HLA alleles and HCC. However, the association between HLA-DQB1 alleles and HCC in different ethnic populations is controversial. As many conflicting reports have been published to date, we conducted a systematic review of all the relevant studies published in the literature to evaluate the association between HLA-DQB1 alleles and HCC. Our principal objectives were to clarify the specific HLA-DQB1 allele families or alleles that are associated with the risk of HCC development.

MATERIALS AND METHODS

Literature search

Electronic databases (PubMed, EMBASE, Cochrane Library and China National Knowledge Infrastructure) were used to search for all genetic association studies evaluating the HLA-DQB1 polymorphism and HCC in humans in all languages up to January 2010. The search strategy was based on combinations of the key words, "HLA" or "human leukocyte antigen" and "hepatocellular carcinoma" or "HCC", and was not restricted by period. We also did a full manual search from the bibliographies of selected papers. In addition, we contacted the authors of studies containing relevant information, who did not report the results necessary for this analysis. Unpublished data were also accepted if an abstract was available and further information was obtained from the authors.

Inclusion and exclusion criteria

In the meta-analysis, the following inclusion criteria were set and reviewed by two independent investigators: (1) an independent case-control study; (2) studies with similar purpose and statistical methods; (3) studies providing

enough information to calculate the odds ratio (OR); (4) HLA-DQB1 alleles were molecularly typed (high or low resolution level); and (5) the diagnosis of HCC was based on at least one of the following criteria: typical histological characteristics or serum α -fetoprotein (AFP) levels higher than 400 ng/mL together with radiological findings (ultrasound and CT) consistent with HCC.

The following exclusion criteria were set: (1) incomplete raw data; (2) repetitive reports (if more than one version of the same study was retrieved, only the most recent was used); and (3) materials and methods were not well-described and reliable.

Although assessment of study quality is considered important for systematic reviews and meta-analyses, scoring methods have been considered problematic^[6] and may not accurately assess the quality measures of interest^[7]. Therefore, we used reliability of patient selection, molecular typing method, and statistical analysis method as quality variables.

The frequency of HLA-DQB1 alleles varies according to ethnic and racial background, with some alleles being extremely rare. Therefore, articles were not required to identify all alleles for inclusion.

Data extraction

The studies were independently evaluated by two researchers (Xin YN and Lin ZH). Discrepancies in the evaluations of studies were resolved by discussion between the reviewers.

The following data were collected from each study: authors, publication year, journal, publication type and language, HLA genotyping method, allele genotype, number of cases and controls, definitions used for HCC, HCC sample description, control sample description (if there was more than one control group, we choose the healthy group as the control group in order to minimize the confounder). The main features of the trials included in the meta-analysis are shown in Table 1.

Statistical analysis

Homogeneity was calculated by Cochran's Q test ($\alpha = 0.05$). If the results of the Q test had no significant heterogeneity, the Mantel-Haenszel fixed-effect model (Peto method) was used for the combined data. If the results of the Q test had significant heterogeneity, the Dersimonian-Laird random-effects model (DL method) was used for the combination of data^[8]. A pooled OR was presented as a standard plot with 95% CIs. In the absence of heterogeneity, the two methods provided identical results. As a measure of association between HCC and HLA-DQB1 alleles, we combined ORs with 95% CIs stratified by gene subtype of patients and controls in a study. Funnel plots and the Egger's regression asymmetry test were used to evaluate publication bias^[9]. We performed a sensitivity analysis to assess the stability of the results by sequential omission of individual studies. All P values presented are two-tailed. The analyses were performed using Revman 5.0 provided by the Cochrane Collaboration Internet.

Table 1 Characteristics of studies included in the meta-analysis

Study	Country/ region	No. of HCC (M/F), age (yr)	No. of controls (M/F), age (yr)	No. of DQB1 alleles studied	Type of control	HLA genotyping method
Li <i>et al</i> ^[10] , 1995	Hong Kong	49 (-/-), ND	78 (-/-), ND	11	Healthy	PCR-SSP
Donaldson <i>et al</i> ^[11] , 2001	Hong Kong	84 (79/5), 55	124 (-/-), ND	10	Healthy	PCR-SSOP
De Re <i>et al</i> ^[12] , 2004	Italy	29 (-/-), ND	144 (-/-), ND	5	Healthy	PCR-SSP
López-Vázquez <i>et al</i> ^[13] , 2004	Spain	46 (27/19), 62 ± 8	48 (19/29), 56 ± 12	10	HCV carriers	PCR-SSOP
Liu <i>et al</i> ^[14] , 2007	China	78 (63/15), 58.8 ± 11.5	100 (-/-), ND	19	Healthy	PCR-SSP
El-Chennawi <i>et al</i> ^[15] , 2008	Egypt	50 (45/5), 51.16 ± 6.16	50 (44/6), 48.88 ± 9.22	5	Healthy	PCR-SSP
Pan ^[16] , 2009	China	62 (52/10), 53.58	50 (29/21), 30.12	8	Healthy	PCR-SSP

HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen; ND: Not described; PCR-SSOP: Polymerase chain reaction-sequence-specific oligonucleotides probes; PCR-SSP: Polymerase chain reaction-sequence specific primer; (-/-): No data was mentioned in the included studies.

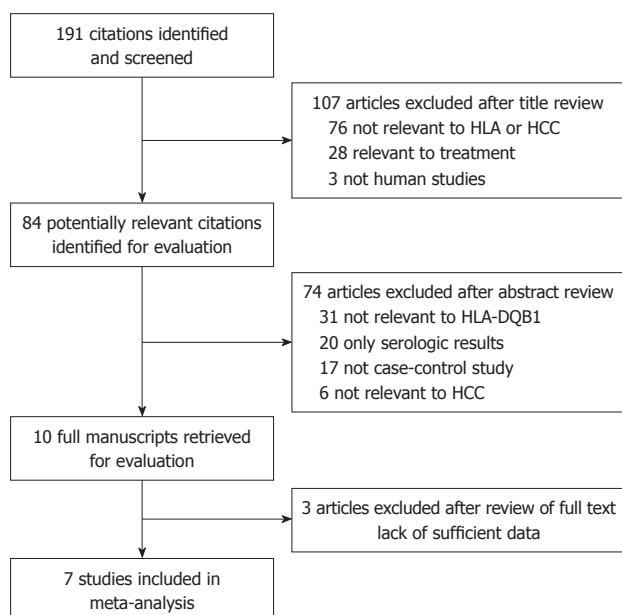


Figure 1 Flow diagram of study identification. HCC: Hepatocellular carcinoma; HLA: Human leukocyte antigen.

RESULTS

Literature assessment

Figure 1 shows the flow diagram of publications identified by the literature search. The search strategy allowed us to identify 60 studies for potential inclusion in the meta-analysis. Only 7 case-control studies relating to HLA-DQB1 alleles polymorphism and susceptibility to HCC qualified on the basis of our selection criteria^[10-16]. A total of 991 subjects were studied (398 patients and 593 controls) (Table 1). Two of these studies were conducted in mainland China, two in Hong Kong, and one each in Italy, Egypt, and Spain. The diagnosis of HCC was based on at least one of the following criteria: typical histological characteristics or serum AFP levels higher than 400 ng/mL together with radiological findings (ultrasound and/or CT) consistent with HCC. The control group of six studies consisted of healthy individuals, while only one study used HCV carriers as the control group^[13]. Records of mean or median age and sex were incomplete in 4/7 reports. HIV status was determined in two reports^[12,15].

HLA-DQB1 alleles were considered only when they were classified in at least two independent studies.

HLA-DQB1 alleles were molecularly typed (high or low resolution level). Six studies used high resolution molecular typing for HLA, while the Italian study used both high and low resolution molecular typing for HLA. Low resolution molecular typing methods for HLA could not identify the specific alleles. Accurate methods for HLA class II typing should involve the combination of polymerase chain reaction (PCR)-sequence-specific oligonucleotides probes, PCR-sequence specific primer and PCR-single strand conformation polymorphism^[17].

Association between HLA-DQB1 alleles and HCC

A total of five HLA-DQB1 allele families and 13 specific alleles were extracted from the studies to investigate their association with HCC. Six specific alleles were excluded because each was identified in only one study.

Among the allele families, two (DQB1*02 and DQB1*03) were significantly associated with risk of HCC. In the meta-analysis, the overall frequency of DQB1*02 was 32.9% (131/398) in HCC, and 24.5% (145/593) in controls. The heterogeneity test indicated that the variation of trial-specific ORs was statistically significant ($\chi^2 = 15.28$, $P = 0.02$ and < 0.05); the random-effect method was used to combine the results. The combined OR was 1.78 (95% CI: 1.05-3.03) and was statistically significant ($P = 0.03$ and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the studies by Li *et al*^[10], López-Vázquez *et al*^[13], Liu *et al*^[14] and El-Chennawi *et al*^[15], which produced a non-significant association. Overall, the frequency of DQB1*03 was 50.0% (199/398) in HCC and 62.7% (372/593) in controls. The heterogeneity test indicated that the variation of trial-specific ORs was not statistically significant ($\chi^2 = 11.53$, $P = 0.07$ and > 0.05). The fixed-effect method was used to combine the results. The combined OR was 0.65 (95% CI: 0.48-0.89) and was statistically significant ($P = 0.007$ and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the study by De Re *et al*^[12], which produced a non-significant association. Statistics calculated for each study about DQB1*03 are shown in the forest plot (Figures 2 and 3). Meta-analysis for another 3 HLA-DQB1 allele

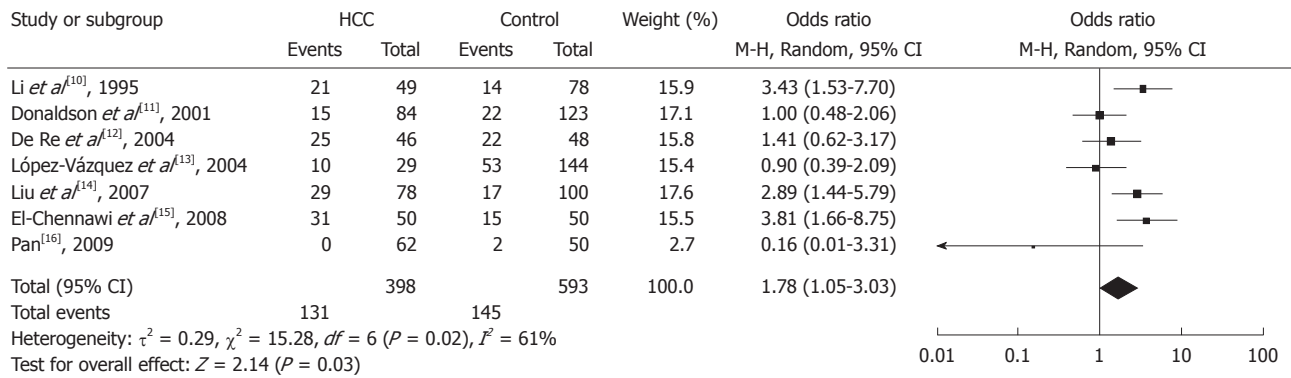


Figure 2 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*02 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

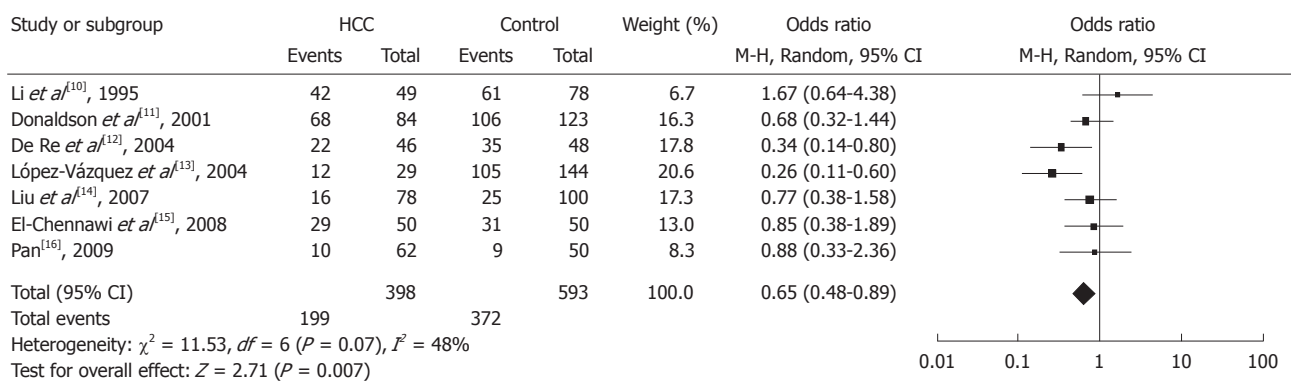


Figure 3 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*03 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

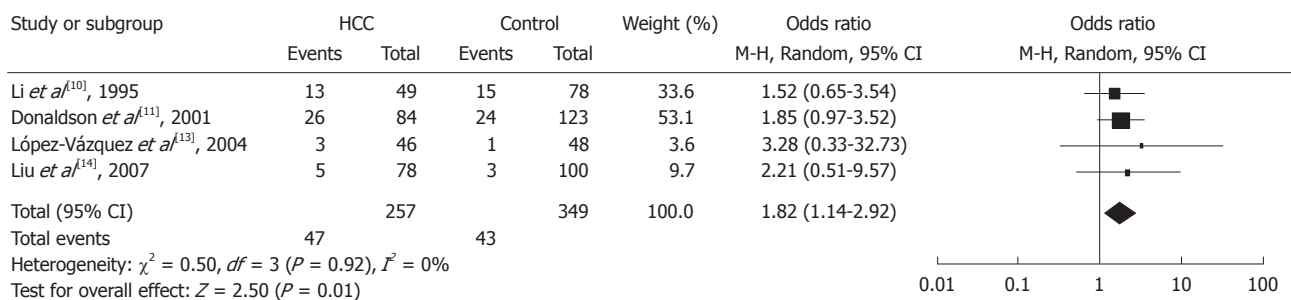


Figure 4 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*0502 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

families (DQB1*04, DQB1*05 and DQB1*06) was carried out, but did not show any statistical effect.

Among the specific alleles, two (DQB1*0502 and DQB1*0602) were significantly associated with risk of HCC. In the meta-analysis, the overall frequency of HLA-DQB1*0502 allele was 18.3% (47/257) in HCC, and 12.3% (43/349) in the controls. The heterogeneity test indicated that the variation of trial-specific ORs was not statistically significant ($\chi^2 = 0.50$, $P = 0.92$ and > 0.05); the fixed-effect method was used to combine the results. The combined OR was 1.82 (95% CI: 1.14-2.92), and was statistically significant ($P = 0.01$ and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the study by Donaldson *et al.*^[11], which produced a non-significant association. Overall, the frequency of HLA-DQB1*0602 allele was 23.7% (41/173) in HCC and 31.4% (71/226) in controls. The heterogeneity test indicated that the variation of trial-specific ORs was not statistically significant ($\chi^2 = 1.26$, $P = 0.53$ and > 0.05); the fixed-effect method was used to combine the results. The combined OR was 0.58 (95% CI: 0.36-0.95) and was statistically significant ($P = 0.03$ and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the study by Liu *et al.*^[14], which produced a non-significant association. Statistics calculated for each study of DQB1*0502 and DQB1*0602 are shown in the forest plot (Figures 4 and 5). Meta-analysis for another 11 HLA-DQB1 specific alleles

et al.^[11], which produced a non-significant association. Overall, the frequency of HLA-DQB1*0602 allele was 23.7% (41/173) in HCC and 31.4% (71/226) in controls. The heterogeneity test indicated that the variation of trial-specific ORs was not statistically significant ($\chi^2 = 1.26$, $P = 0.53$ and > 0.05); the fixed-effect method was used to combine the results. The combined OR was 0.58 (95% CI: 0.36-0.95) and was statistically significant ($P = 0.03$ and < 0.05). In sensitivity analysis, the exclusion of individual studies did not change this significant result, except for the study by Liu *et al.*^[14], which produced a non-significant association. Statistics calculated for each study of DQB1*0502 and DQB1*0602 are shown in the forest plot (Figures 4 and 5). Meta-analysis for another 11 HLA-DQB1 specific alleles

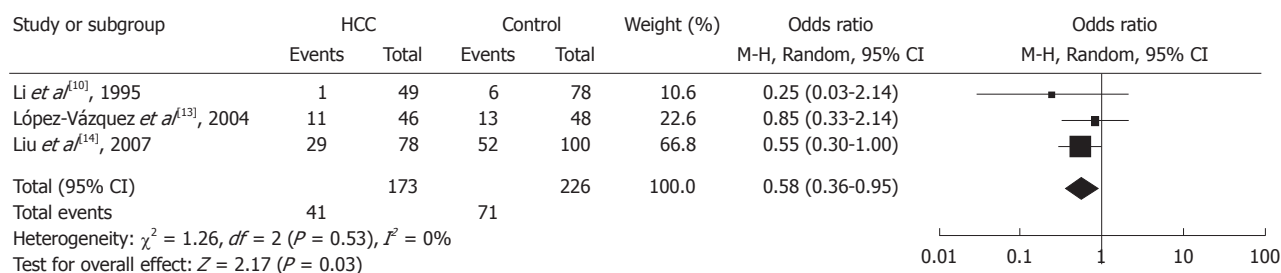


Figure 5 Meta-analysis forest plot of included studies on the association between human leukocyte antigen-DQB1*0602 allele and hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

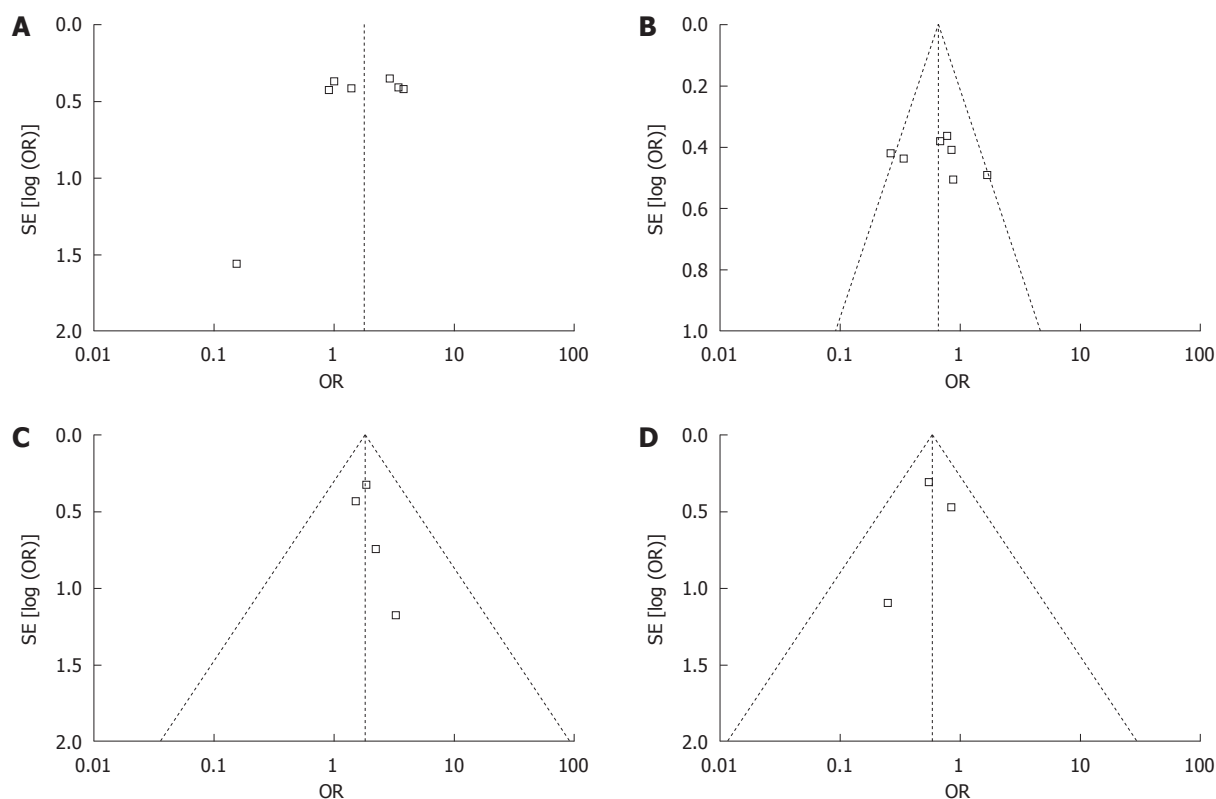


Figure 6 The funnel plot to detect publication bias of each study for DQB1*02, DQB1*03, DQB1*0502 and DQB1*0602. Each point represents a separate study for the indicated association. A: Funnel plot analysis of human leukocyte antigen (HLA)-DQB1*02 allele and hepatocellular carcinoma (HCC) to detect publication bias; B: Funnel plot analysis of HLA-DQB1*03 allele and HCC to detect publication bias; C: Funnel plot analysis of HLA-DQB1*0502 allele and HCC to detect publication bias; D: Funnel plot analysis of HLA-DQB1*0602 allele and HCC to detect publication bias.

(DQB1*0201, DQB1*0301, DQB1*0302, DQB1*0303, DQB1*0401, DQB1*0402, DQB1*0501, DQB1*0503, DQB1*0601, DQB1*0603 and DQB1*0604) was carried out, but did not show any statistical relationships.

Our meta-analysis of seven studies revealed that DQB1*02 and DQB1*0502 were risk factors for HCC, while DQB1*03 and DQB1*0602 were protective factors. These results suggest that patients with DQB1*02 and DQB1*0502 alleles were at a higher risk of developing HCC than those with DQB1*03 and DQB1*0602 alleles. These analyses were based on the data from seven studies irrespective of the ethnicity of the study populations. The shape of the funnel plot to detect publication bias of each study for DQB1*02, DQB1*03, DQB1*0502 and DQB1*0602 seemed to be asymmetrical (Figure 6), sug-

gesting that publication bias might affect the findings of our meta-analysis.

DISCUSSION

To our knowledge, this is the first published meta-analysis to comprehensively investigate the association between HLA-DQB1 alleles and HCC. Numerous studies have reported associations of HLA with malignant tumors, such as thyroid cancer, renal cell cancer, Burkett's lymphoma, Kaposi's sarcoma, Hodgkin's disease, nasopharyngeal carcinoma, cervical carcinoma, esophageal carcinoma, gastric cancer, and HCC. However, most of these studies focused on the association between HLA antigen and cancer based on HLA serotyping which has limited reso-

lution, and may be inaccurate for the assignment of many HLA antigens. With the development of DNA-based methods for HLA genotyping, HLA genotyping has become more accurate in the determination of the HLA-DQB1 alleles, and more precise than serotyping in the determination of the peptide-binding domain of MHC class II molecules. Hence, HLA genotyping is being used more frequently in the field of immunogenetics.

Recent studies on the association between HLA-DQB1 allele polymorphisms and HCC have been inconclusive. Pan^[16] reported that some HLA-DQB1 alleles are significantly associated with HCC (DRB1*04 and DQB1*02), while others (DQB1*06) are not. Therefore, it is possible that the DRB1*04 and DQB1*02 alleles might be risk factors for the occurrence of HCC (OR = 4.373 and 3.807, respectively), and DQB1*06 may be a protective allele (OR = 0.259). Lopez-Vazquez *et al.*^[13] confirmed that HLA-DQB1*0301 carriers rarely develop end-stage liver disease (ESLD). This allele may, therefore, be considered to be a good prognostic factor. De Re *et al.*^[12] have shown that the DQB1*0301 allele played a major protective role in patients with HCV-associated HCC. Donaldson *et al.*^[11] observed that the alleles DRB1*1501, DQA1*0302 and to a lesser extent DQB1*0302 appeared to confer resistance to HCC.

The current meta-analysis indicates that specific HLA-DQB1 alleles are associated with HCC. Among the 5 family alleles, DQB1*02 and DQB1*03 were significantly associated with risk of HCC. The combined OR was 1.78 (95% CI: 1.05-3.03) and 0.65 (95% CI: 0.48-0.89), respectively. Among the 13 specific alleles, DQB1*0502 and DQB1*0602 were significantly associated with risk of HCC. The combined OR was 1.82 (95% CI: 1.14-2.92) and 0.58 (95% CI: 0.36-0.95), respectively. These results suggest that DQB1*02 and DQB1*0502 alleles may have a higher risk for HCC, while DQB1*03 and DQB1*0602 alleles may have a protective effect for HCC.

The DQB1*02 allele family is negatively associated with the incidence of breast cancer in the Tunisian population^[18], while the DQB1*03 allele family is associated with susceptibility to cervical carcinoma in Europe (OR = 3.03)^[19], cutaneous T-cell lymphoma in USA (OR = 2.7)^[20], and HCV-related non-Hodgkin's lymphoma in Italy (OR = 0.23)^[12]. The DQB1*0502 allele is also associated with the susceptibility to Sezary syndrome of cutaneous T-cell lymphoma in USA (OR = 7.75)^[20], while the DQB1*0602 allele is associated with an increased gastric cancer risk in Taiwan (OR = 2.79)^[21] and increased distal gastric cancer risk in southern European population (OR = 4.82)^[22]. DQB1*0602 also appears to be a candidate protective allele for testicular germ cell carcinoma in the Japanese population (RR = 0.26, $P = 0.02$)^[23]. The mechanisms for these effects are unknown, but larger-scale studies may confirm this observation.

Human tumor cells express diverse types of antigens, depending on the etiology and pathogenesis of the disease^[24]. Because tumor development is preceded by chronic inflammation, immune responses, whether towards the infectious agent itself or against tumor antigens, may

be critical for development of tumor. Different types of HLA molecules have different capability of binding and presenting tumor antigens. The association of specific HLA alleles with susceptibility or resistance to malignant tumors is probably attributable to a direct involvement of the HLA molecules as an antigen presenter or possibly due to a neighboring linked gene^[14].

Several other points should be considered when interpreting the results of our study. Most studies did not control for the matching variables in the analysis, and the risk for HCC was not controlled for possible confounders such as HBV or HCV. These observational studies are more prone to bias than randomized clinical trial (RCT) studies. The shape of the funnel plot seemed to be asymmetrical, suggesting that publication bias might affect the findings of our meta-analysis.

In conclusion, this meta-analysis reveals that DQB1*02 and DQB1*0502 are risk factors for HCC, while DQB1*03 and DQB1*0602 are protective factors. These findings are consistent with the previous studies about the association of HLA alleles and other cancers. In the present study, no attempt has been made to account for the potential confounding influence of cirrhosis in the development of HCC, although future studies of HLA should take this into consideration.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide and about 600 000 patients died from the disease annually. Human leukocyte antigen (HLA) polymorphism is implicated in conferring genetic susceptibility to a large number of immune-mediated diseases, including some cancers.

Research frontiers

The clustering of HCC within families raises the possibility that genetic factors are involved in susceptibility to HCC. Much attention has been paid to the potential role of HLA in the pathogenesis of HCC in an attempt to uncover the underlying mechanisms. However, the relationship between HLA-DQB1 and HCC remains controversial and no meta-analysis has been conducted.

Innovations and breakthroughs

This is the first meta-analysis which systemically studied the relationship between HLA-DQB1 and HCC susceptibility, and suggested that specific HLA-DQB1 allele families and alleles might influence the susceptibility or resistance to HCC, which needs further investigations.

Applications

The study concludes that DQB1*02 and DQB1*0502 are risk factors for HCC, while DQB1*03 and DQB1*0602 are protective factors, which provides an insight into the role of HLA-DQB1 in the pathogenesis of HCC. Furthermore, these findings are meaningful to early diagnosis and provide a new strategic approach to the prevention of HCC.

Terminology

Meta-analysis is a means of increasing the effective sample size under investigation through the pooling of data from individual association studies, thus enhancing the statistical power of the analysis.

Peer review

This is a very interesting meta-analytic study dealing with an important topic in HCC. The described analysis has been performed with precision and accuracy.

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Neoadjuvant sorafenib combined with gemcitabine plus oxaliplatin in advanced hepatocellular carcinoma

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Abstract

This paper reports the first case of a patient with hepatocellular carcinoma with lymph node metastasis treated by sorafenib combined with gemcitabine plus oxaliplatin, with a partial response and normalization of α fetoprotein, which allowed curative surgery. The potential synergy between these three drugs needs to be confirmed, and is currently being investigated in a randomized phase II trial.

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Key words: Hepatocellular carcinoma; Gemcitabine; Oxaliplatin; Sorafenib; Neoadjuvant therapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality^[1]. Underlying cirrhosis is present in 90% of cases. Its prognosis remains poor with lots of advanced forms, and there is no curative therapeutic approach.

Since 2007, sorafenib has been the standard treatment for first-line therapy of advanced or metastatic disease that is not eligible for intra-arterial chemo-embolization. It has been shown to significantly improve progression-free and overall survival^[2]. However, sorafenib is not able to induce any objective response and tumor shrinkage that allows secondary surgery. Its role in neoadjuvant therapy has not been established either.

Although HCC has been documented as a chemoresistant tumor, some chemotherapeutic regimens, such as gemcitabine plus oxaliplatin, have shown some interesting efficacy results, with objective response rates around 20% and disease control rates > 50% in phase II trials^[3-5]. Here, we report what is believed to be the first case of a patient with advanced HCC who received sorafenib together with gemcitabine plus oxaliplatin, which allowed secondary curative surgery.

CASE REPORT

A 61-year-old man, working as a radiologist, was admitted

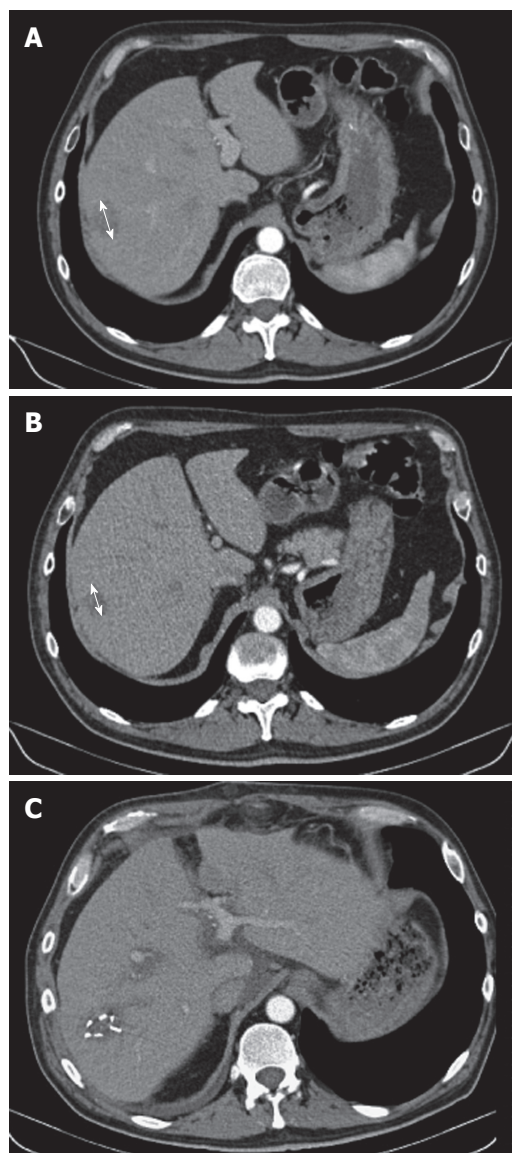


Figure 1 Computed tomography scan. A: A 4.5-cm hypervascular hepatic tumor (arrow) that occupied the sixth hepatic segment, without signs of underlying cirrhosis; B: After eight cycles of gemcitabine and oxaliplatin combined with sorafenib, we observed tumor shrinkage of > 40% (arrow), as compared to baseline assessment; C: Secondary surgery was performed: hemostatic clips were still visible on this postoperative CT scan.

for a liver mass that was detected by ultrasonography that was performed for abdominal pain, in December 2009. He had a history of cholecystectomy and post-transfusional hepatitis C, which was treated effectively by interferon and ribavirin therapy in 1998. Clinically, the ECOG performance status was 0, the patient weighed 94 kg, his height was 188 cm, and he had no symptoms except for moderate abdominal pain that was controlled with class II analgesics. In January 2010, Abdominal computed tomography (CT) showed a 4.5-cm hypervascular hepatic tumor that occupied the sixth hepatic segment, without signs of underlying cirrhosis (Figure 1A). There were also two metastatic lymph nodes (MLNs): one celiac measuring 4 cm, and one measuring 3 cm located in the liver hilum (Figure 2A). Laboratory data showed normal blood

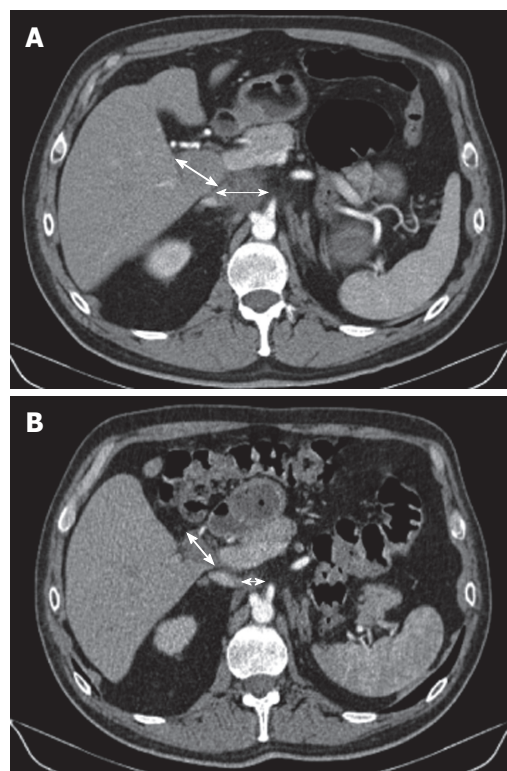


Figure 2 Computed tomography scan. A: Initially, there were two metastatic lymph nodes (MLNs): one celiac (thin arrow) measuring 4 cm and one measuring 3 cm located in the liver hilum (thick arrow); B: Computed tomography scan taken after the end of the eight cycles of gemcitabine plus oxaliplatin combined with sorafenib, showed that the MLNs (thin arrow for celiac node and thick arrow for hilar node) decreased in size dramatically with a more necrotic aspect, which allowed secondary surgery followed by radiotherapy.

cell and platelet counts, liver enzymes, bilirubin and albumin levels. α -fetoprotein (AFP) was markedly increased at 2572 ng/mL (upper limit of normal = 8). Esophago-gastroduodenoscopy did not show gastroesophageal varices or other upper gastrointestinal tract abnormalities.

Fine needle echoendoscopic aspiration from celiac lymph nodes was performed and showed a typical trabecular liver carcinoma. Evaluation by a multidisciplinary team concluded that the primary tumor was theoretically resectable in this patient, who had an excellent performance status, without underlying cirrhosis, and who wished an aggressive therapeutic approach. However, because there were MLNs, initial surgery was rejected and intensive systemic therapy with sorafenib combined with gemcitabine plus oxaliplatin was initiated. He received four cycles of the GEMOX regimen (gemcitabine 1 g/m² on day 1 and oxaliplatin 100 mg/m² on day 2), as previously described^[3]. This treatment was repeated every 2 wk and sorafenib was taken every day (400 mg twice daily). No grade 3/4 toxicities were observed. Grade 1 diarrhea, thrombopenia and neurotoxicity were reported, together with grade 2 asthenia after the fourth cycle. Efficacy assessment showed a decrease of AFP from 2572 to 97 ng/mL. CT scan showed tumor shrinkage of 25% in the liver (Figure 1B), and a slight decrease in MLN size, which were more necrotic (Figure 2B). A new multidisciplinary

evaluation was performed and it was decided to continue the same therapeutic schedule for more cycles. Second efficacy evaluation showed normalization of AFP levels (2.7 ng/mL) and tumor volume reduction of around 40% (Figure 2A), as compared to baseline assessment.

Surgery was then decided upon, which consisted of removing the primary tumor, together with satellite adenopathies. The sites were marked by surgical clips (Figure 1C) to allow for postoperative radiotherapy (RT).

Pathological examination of the specimens showed a well-differentiated HCC that measured 2.5 cm, without underlying cirrhosis (METAVIR score A1F2). All resection margins were negative, and only one lymph node of five removed was metastatic, which was located in the liver hilum and measured 2 cm, with a necrotic center. There were no postoperative complications except for exacerbation of oxaliplatin-induced neurosensory toxicity, as previously described^[6,7]. Global status of the patient improved and postoperative RT of MLNs was performed, by delivering 46 Gy over 5 wk, without any side effects. Complementary adjuvant chemotherapy, with combined sorafenib and gemcitabine for 3 wk in four, was started for 3 mo, and is currently ongoing. Oxaliplatin was not reintroduced because of persistent grade 2 neurosensory toxicity.

DISCUSSION

The role of chemotherapy in HCC patients is still a matter of debate. There is no clear evidence for efficacy of any tested schedule in the adjuvant (or neoadjuvant) setting, as reviewed by a recent Cochrane study^[8]. However, many oncologists are using systemic chemotherapy for their HCC patients in a palliative setting. Doxorubicin, which was considered as potential standard chemotherapy 20 years ago, seems to be toxic and does not convincingly improve survival over that with supportive care^[9]. Recent studies have suggested that oxaliplatin and gemcitabine could be interesting agents, alone or in combination, even if there has been no phase III trial to confirm their efficacy. The choice of gemcitabine-oxaliplatin combinations in this disease is based on: (1) synergy between the two drugs and their sequence dependency (gemcitabine followed by oxaliplatin is most active in preclinical models)^[10]; (2) clinical activity of gemcitabine alone and of 5-fluorouracil/oxaliplatin combination in patients with HCC in phase II trials^[11,12]; and (3) lack of renal and hepatic oxaliplatin toxicity in cirrhotic patients, unlike doxorubicin. The GEMOX combination has thus been evaluated in many phase II studies with promising results in term of response rate and survival^[3-5,13]. Patients with complete response have been described by ourselves and others^[14].

In the pivotal SHARP trial, response rate with sorafenib was particularly low (2.3%) and not significantly different from placebo^[2]. To the best of our knowledge, only one patient receiving sorafenib and eligible for secondary surgery has been reported in the literature^[15]. Sorafenib does

not seem to be a candidate drug for tumor downstaging that allows secondary surgery. However, the antiangiogenic effect of sorafenib seems to modify HCC vascularization, by normalizing tumor vessels^[16]. This vascular normalization has been described as potentially favoring chemotherapy efficacy through better delivery of the drugs to the tumor site^[17]. Combination of sorafenib and GEMOX could thus lead to synergistic antitumor activity.

The toxicity of the GEMOX regimen has been well described over the past 10 years through 31 phase II and two phase III trials, mainly in pancreatic and biliary tract cancers. Grade 3 neutropenia, thrombocytopenia and neurotoxicity have been reported in 22%, 11% and 10%, respectively, and severe nausea has occurred in 16% of the cases^[18]. These results are similar to those described in the phase II trials that have evaluated this combination in HCC patients with underlying cirrhosis. The elevated liver enzymes and anaphylactic reactions (due to gemcitabine and oxaliplatin, respectively) did not seem more frequent in this population.

In the SHARP trial, diarrhea (grade 2 in 50%, grade 3 in 10%), weight loss, fatigue, hand-foot skin reaction (grade 2 in 30%, grade 3 in 8%), and hypophosphatemia were the most frequent toxicities associated with sorafenib. To date, no trial has assessed GEMOX plus sorafenib combination therapy.

Adjuvant RT after resection of MLNs from HCC is not common. To the best of our knowledge, only two Asian cases have been described in the literature^[19,20]. However, three retrospective studies have demonstrated its role in local control of MLNs from HCC that is not eligible for surgery, but is present in patients with good performance status. RT doses range from 40 to 60 Gy, and could prolong overall survival^[21-23]. For example, in Zeng's retrospective study on 125 patients, partial and complete responses were observed in 37.1% and 59.7% of patients, respectively. The median survival was 9.4 mo^[23]. However, these results need to be confirmed. To avoid unnecessary toxicity, the RT dose chosen in the present case did not exceed 50 Gy^[21].

In conclusion, this case is an example of successful multimodal aggressive treatment for advanced HCC, which combined for the first time, GEMOX and sorafenib followed by surgery and adjuvant radiotherapy and chemotherapy. GEMOX + sorafenib combination therapy was well tolerated with only grade 1/2 toxicity that was easily manageable, and gave an objective response, together with a dramatic decrease in AFP levels. These promising results need to be confirmed and GEMOX/sorafenib combination therapy is currently being evaluated in an ongoing randomized phase II trial and compared to sorafenib alone.

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MEETINGS

Events Calendar 2011

January 14-15, 2011

AGA Clinical Congress of
Gastroenterology and Hepatology:
Best Practices in 2011 Miami, FL
33101, United States

January 20-22, 2011

Gastrointestinal Cancers Symposium
2011, San Francisco, CA 94143,
United States

January 27-28, 2011

Falk Workshop, Liver and
Immunology, Medical University,
Franz-Josef-Strauss-Allee 11, 93053
Regensburg, Germany

January 28-29, 2011

9. Gastro Forum München, Munich,
Germany

February 4-5, 2011

13th Duesseldorf International
Endoscopy Symposium,
Duesseldorf, Germany

February 13-27, 2011

Gastroenterology: New Zealand
CME Cruise Conference, Sydney,
NSW, Australia

February 17-20, 2011

APASL 2011-The 21st Conference of
the Asian Pacific Association for the
Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011

Canadian Digestive Diseases Week
2011, Vancouver, BC, Canada

February 24-26, 2011

Inflammatory Bowel Diseases
2011-6th Congress of the European
Crohn's and Colitis Organisation,
Dublin, Ireland

February 24-26, 2011

2nd International Congress on
Abdominal Obesity, Buenos Aires,
Brazil

February 24-26, 2011

International Colorectal Disease
Symposium 2011, Hong Kong, China

February 26-March 1, 2011

Canadian Digestive Diseases Week,
Westin Bayshore, Vancouver, British
Columbia, Canada

February 28-March 1, 2011

Childhood & Adolescent Obesity:

A whole-system strategic approach,
Abu Dhabi, United Arab Emirates

March 3-5, 2011

42nd Annual Topics in Internal
Medicine, Gainesville, FL 32614,
United States

March 7-11, 2011

Infectious Diseases: Adult Issues
in the Outpatient and Inpatient
Settings, Sarasota, FL 34234,
United States

March 14-17, 2011

British Society of Gastroenterology
Annual Meeting 2011, Birmingham,
England, United Kingdom

March 17-19, 2011

41. Kongress der Deutschen
Gesellschaft für Endoskopie und
Bildgebende Verfahren e.V., Munich,
Germany

March 17-20, 2011

Mayo Clinic Gastroenterology &
Hepatology 2011, Jacksonville, FL
34234, United States

March 18, 2011

UC Davis Health Informatics:
Change Management and Health
Informatics, The Keys to Health
Reform, Sacramento, CA 94143,
United States

March 25-27, 2011

MedicRes IC 2011 Good Medical
Research, Istanbul, Turkey

March 26-27, 2011

26th Annual New Treatments in
Chronic Liver Disease, San Diego,
CA 94143, United States

April 6-7, 2011

IBS-A Global Perspective, Pfister
Hotel, 424 East Wisconsin Avenue,
Milwaukee, WI 53202, United States

April 7-9, 2011

International and Interdisciplinary
Conference Excellence in Female
Surgery, Florence, Italy

April 15-16, 2011

Falk Symposium 177, Endoscopy
Live Berlin 2011 Intestinal Disease
Meeting, Stauffenbergstr. 26, 10785
Berlin, Germany

April 18-22, 2011

Pediatric Emergency Medicine:
Detection, Diagnosis and Developing

Treatment Plans, Sarasota, FL 34234,
United States

April 20-23, 2011

9th International Gastric Cancer
Congress, COEX, World Trade
Center, Samseong-dong, Gangnam-
gu, Seoul 135-731, South Korea

April 25-27, 2011

The Second International Conference
of the Saudi Society of Pediatric
Gastroenterology, Hepatology &
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011

Neurology Updates for Primary
Care, Sarasota, FL 34230-6947,
United States

April 28-30, 2011

4th Central European Congress of
Surgery, Budapest, Hungary

May 7-10, 2011

Digestive Disease Week, Chicago, IL
60446, United States

May 12-13, 2011

2nd National Conference Clinical
Advances in Cystic Fibrosis, London,
England, United Kingdom

May 19-22, 2011

1st World Congress on Controversies
in the Management of Viral Hepatitis
(C-Hep), Palau de Congressos de
Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011

22nd European Society of
Gastrointestinal and Abdominal
Radiology Annual Meeting and
Postgraduate Course, Venice, Italy

May 25-28, 2011

4th Congress of the Gastroenterology
Association of Bosnia and
Herzegovina with international
participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011

The International Digestive Disease
Forum 2011, Hong Kong, China

June 13-16, 2011

Surgery and Disillusion XXIV
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011

International Scientific Conference
on Probiotics and Prebiotics-
IPC2011, Kosice, Slovakia

June 22-25, 2011

ESMO Conference: 13th World
Congress on Gastrointestinal Cancer,
Barcelona, Spain

June 29-2, 2011

XI Congreso Interamericano
de Pediatría "Monterrey 2011",
Monterrey, Mexico

September 2-3, 2011 Falk Symposium

178, Diverticular Disease, A Fresh
Approach to a Neglected Disease,
Gürzenich Cologne, Martinstr. 29-37,
50667 Cologne, Germany

September 10-11, 2011

New Advances in Inflammatory
Bowel Disease, La Jolla, CA 92093,
United States

September 10-14, 2011

ICE 2011-International Congress of
Endoscopy, Los Angeles Convention
Center, 1201 South Figueroa Street
Los Angeles, CA 90015,
United States

September 30-October 1, 2011

Falk Symposium 179, Revisiting
IBD Management: Dogmas to be
Challenged, Sheraton Brussels
Hotel, Place Rogier 3, 1210 Brussels,
Belgium

October 19-29, 2011

Cardiology & Gastroenterology |
Tahiti 10 night CME Cruise, Papeete,
French Polynesia

October 22-26, 2011

19th United European
Gastroenterology Week, Stockholm,
Sweden

October 28-November 2, 2011

ACG Annual Scientific Meeting &
Postgraduate Course, Washington,
DC 20001, United States

November 11-12, 2011

Falk Symposium 180, IBD 2011:
Progress and Future for Lifelong
Management, ANA Interconti Hotel,
1-12-33 Akasaka, Minato-ku, Tokyo
107-0052, Japan

December 1-4, 2011

2011 Advances in Inflammatory
Bowel Diseases/Crohn's & Colitis
Foundation's Clinical & Research
Conference, Hollywood, FL 34234,
United States



GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the “priority” and “copyright” of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers’ names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid

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The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systematically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensus and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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In the interests of transparency and to help reviewers assess any potential bias, WJG requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/..."; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 vs 3.61 ± 1.67 , $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be

Instructions to authors

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar 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 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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- 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

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- 12 **Breedlove GK**, Schorheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 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

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- 15 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/index.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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