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EDITORIAL

Value of colonoscopy for prediction of prognosis in patients with ulcerative colitis

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disorder characterized by exacerbations and remissions. Some UC patients remain refractory to conventional medical treatment while, in others, the effectiveness of drugs is limited by side-effects. Recently, cyclosporine and leukocyte removal therapy have been used for refractory UC patients. To predict the efficacy of these therapies is important for appropriate selection of treatment options and for preparation for colectomy. Endoscopy is the cornerstone for diagnosis and evaluation of UC. Endoscopic parameters in patients with severe or refractory UC may predict a clinical response to therapies, such as cyclosporine or leukocyte removal therapy. As for the patients with quiescent UC, relapse of UC is difficult to predict by routine colonoscopy. Even when routine colonoscopy suggests remission and a normal mucosal appearance, microscopic abnormalities may persist and relapse may occur later. To more accurately identify disease activity and to predict exacerbations in UC patients with clinically inactive disease is important for deciding whether medical treatment should be maintained. Magnifying colonoscopy is useful for the evaluation of disease activity and for predicting relapse in patients with UC.

Key words: Ulcerative colitis; Colonoscopy; Prediction of outcome

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disorder characterized by diffuse mucosal inflammation of the colorectum with exacerbations and remissions[1-5]. Approximately 15% of patients experience a severe exacerbation requiring hospital admission at some time during their illness[3,6]. The purpose of treatments for patients with ulcerative colitis is achieving remission and maintaining quiescence of the disease. Patients with UC must rely on multiple medications to control their symptoms, including aminosalicylates, corticosteroids and purine analogs. Although decades of clinical experience in the management of UC have allowed the optimization of approaches to the induction and maintenance of remission, some patients remain refractory to conventional medical treatment and the effectiveness of these drugs may be limited by side-effects[7-11]. The use of immunosuppressive agents, including purine analogs, now constitutes a therapeutic modality for the treatment of UC[12]. Although highly effective, a disadvantage of these drugs is the significant delay in their onset of clinical benefit, which limits their utility to the treatment of severe disease.

Although the degree of inflammation as assessed by routine colonoscopy is a reliable parameter of disease activity, discrepancies between colonoscopic appearance and histopathologic abnormalities are sometimes seen in patients with clinically inactive UC (Figure 1). Even when routine colonoscopy suggests remission and a normal mucosal appearance, microscopic abnormalities may persist[13,14] and relapse may occur later[15]. A recently developed high-resolution video-magnifying colonoscope has enabled the observation of pit patterns on the

surface of the colorectal mucosa. This in turn allows an understanding of the morphological relationship between the pit patterns detected colonoscopically and the crypts observed histopathologically[16-20]. As far back as 1980, Poulsen *et al*[21] examined biopsy specimens from the rectal mucosa of UC patients under a stereomicroscope and found microstructural abnormalities in the mucosal surface in almost every patient, as well as a close correlation between stereomicroscopic features and the clinical disease activity, sigmoidoscopic findings, and histologic activity of the disease.

Here, we discuss endoscopic factors predictive of the efficacy of therapies in patients with intractable UC, and endoscopic factors that may predict the probability of subsequent disease relapse in UC patients in remission. We will reconsider the value of endoscopy when we treat UC patients.

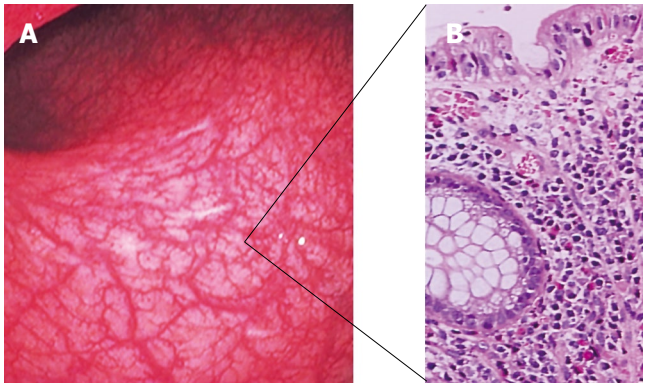

Figure 1 A case of inactive UC. A discrepancy is seen between an endoscopic and a histologic finding. **A**: A routine colonoscopy finding. It shows an almost normal mucosal appearance; **B**: A histologic finding. It shows an intense infiltration of mononuclear cells and neutrophils.

ENDOSCOPIC PREDICTORS OF RESPONSE TO THERAPIES IN PATIENTS WITH REFRACTORY ULCERATIVE COLITIS

In recent years, steroid-refractory cases of UC have been successfully treated by adding intravenous cyclosporine to the glucocorticosteroids. Cyclosporine is a lipophilic cyclic peptide that interrupts the cellular immune response by blocking interleukin 2 productions by T cells. Uncontrolled studies show that approximately 80% of patients with severe UC refractory to glucocorticosteroid treatment respond to cyclosporine therapy[22,23]. The use of cyclosporine is, however, associated with considerable morbidity. Serious complications such as *Pneumocystis carinii* pneumonia and seizures have occurred in as many as 12% of patients in large series, and deaths have been reported[24-26]. Less serious, but nevertheless troubling, side-effects including hypertension, liver and renal impairment, tremor, paresthesia and headache, occur in up to 50% of patients[23,25-27]. It would be useful to define factors predictive of response to cyclosporine treatment for severe flares of ulcerative colitis, to avoid the side effects as well as reduce the risk of subjecting the patients to increased morbidity and mortality due to needlessly delaying colectomy. However, there has been only limited information as to which factors are associated with a response to cyclosporine that leads to possible avoidance of colectomy in such patients. Rowe *et al* demonstrated that a higher percentage of band neutrophils on admission was predictive of patients who were unlikely to respond to cyclosporine and who would require colectomy[28]. On the other hand, McCormack *et al* showed that the *in vitro* cyclosporine sensitivity of proliferating lymphocytes was predictive of the therapeutic response[29]. Genetic factors of the host are also considered to play a role in UC outcomes. The TT genotype of exon 21 multidrug resistance gene 1 polymorphisms is associated with a higher risk of cyclosporine failure in patients with steroid resistant UC[30]. Our prospective analysis with a logistic regression model, colonoscopic findings predictive of response to intravenous cyclosporine in patients with steroid-resistant ulcerative colitis included the presence of deep and extensive ulcerations, and the absence of mucosal bleeding or poor luminal extensibility (Table 1).

Table 1 Colonoscopic finding predictive of response to intravenous cyclosporine in ulcerative colitis patients

	Responders (n = 17)	Non-responders (n = 9)	Relative risk[1] (Odds ratio)
Deep and extensive ulcerations (yes:no)	8:9	0:9	14.20 ($P < 0.005$)
Mucosal bleeding (yes:no)	5:12	7:2	0.12 ($P < 0.05$)
Poor luminal extensibility (yes:no)	4:13	7:2	0.09 ($P < 0.01$)

[1]Logistic regression analysis.

Findings in active UC include the activation and extravasation of large numbers of granulocytes and monocytes/macrophages into the colonic mucosa[31,32]. These infiltrated leukocytes may cause extensive mucosal tissue injury by releasing degradative proteases[32-34], reactive oxygen derivatives[32,34,35], and pro-inflammatory cytokines[36]. Leukocyte removal therapy is recognized as a second novel strategy for the treatment of steroid-refractory UC, based on the assumption that this non-drug therapy attenuates intestinal inflammation by reducing excess and activated granulocytes, monocytes and lymphocytes from the circulating blood before they reach the inflamed mucosa[37]. Adsorption with beads (granulocytapheresis, GCAP) or filters (leukocytapheresis, LCAP) is most commonly used[38,39]. Several studies have reported the beneficial effects of leukocyte removal therapy on both the induction and maintenance of clinical remission in patients with IBD[40-42], suggesting that it may be a useful adjunct to conventional therapy in patients with active severe UC and those refractory to conventional drugs. Further, leukocyte removal therapy might be an effective first line medication[43]. First UC episode and short disease duration are good predictors of response to leukocyte removal therapy[44]. Steroid-naïve patients respond particularly well to this treatment[42,45]. Patients with deep colonic lesions might be less satisfactory[45]. However, our prospective analysis in patients

with steroid resistant ulcerative colitis did not find any colonoscopic findings predictive of response to leukocyte removal therapy[46]. Further study with a larger population of patients needs to be conducted to define predictors of response to cyclosporine or leukocyte removal therapy, including prolonged outcome, for more appropriate selection of treatment options with these therapies in patients with severe ulcerative colitis.

PREDICTION OF RELAPSE IN PATIENTS WITH QUIESCENT ULCERATIVE COLITIS

Severity in ulcerative colitis is generally assessed using symptoms, laboratory data[47], colonoscopic findings[48-55] and histologic degree of inflammation in the biopsy specimens[15,56-59]. Histopathologic assessment is considered the standard for evaluation of disease activity in patients with ulcerative colitis[60]. The observation under conventional colonoscopy has been regarded as useful for the evaluation of disease activity, since it offers direct observation of mucosal changes, but it still remains controversial whether colonoscopic grade correlates with histopathologic findings. It has been reported that the degree of histologic inflammation within biopsy specimens did not necessarily correlate with endoscopic abnormalities[48,49,61,62]. It is not unusual for routine colonoscopy performed to assess the stage of UC to show quiescent colitis despite the histological persistence of inflammation[48,61,63], which later results in the relapse of colonic inflammation[15]. Hurlstone DP *et al* reported high-frequency ultrasound is a valid adjunctive 'tool' for the trans-mural assessment of the colorectal wall in UC[64]. This technique may aid in the initial diagnosis, and ongoing chronic management of disease.

Matsumoto *et al* reported usefulness of magnifying chromoscopy for the assessment of severity in UC patients[65]. In their study, magnifying colonoscopy was performed in 41 patients with ulcerative colitis, and the findings in the rectum were graded according to network pattern (NWP) and cryptal opening (CO). The clinical, endoscopic and histologic grades of activity were not different between groups divided by the presence or absence of each finding. However, when the two features were coupled, patients with visible NWP and CO had a lower clinical activity index and lower grade of histologic inflammation than those in whom both findings could not be visualized. Furthermore it has been suggested that the presence of branches in surface epithelium may be a factor that predicts future disease relapse[15], and they suggested that altered pattern as defined by magnified colonoscopic views may be predictive of the course of ulcerative colitis[65].

Fujiya *et al* proposed the classification of magnifying colonoscopic findings in patients with ulcerative colitis which is useful for the evaluation of disease activity and for the prediction of periods of remission[66]. The classification was devised as follows: regularly arranged crypt opening, villous-like appearance, minute defects of epithelium (MDE), small yellowish spots (YS), and coral reef-like appearance. The colonoscopic findings by classification were compared with histopathologic findings in 61 patients and the usefulness of the classification for predicting relapse was prospectively analyzed in 18 patients. Under conventional colonoscopic examinations, all areas evaluated as Matts grade 1 had a corresponding histopathologic grade 1. In contrast, most areas assessed as Matts grade 3 or 4 were diagnosed as histopathologic grade 3 or higher. However, grade 2 mucosa had histopathologic findings that varied from quiescent to active disease. These suggest that normal and diseased mucosas are easily recognized by conventional colonoscopy, but it is difficult for conventional colonoscopy to assess the minute mucosal changes that reflect smoldering histopathologic inflammation[48,49,61]. In contrast, under magnifying colonoscopic examinations, 37 (82.2%) of the 45 areas in which regularly arranged crypt openings or a villous-like appearance was detected had a corresponding histopathologic grade 1, and all areas in which MDE, SYS, or the coral reef-like appearance was observed had a corresponding histopathologic grade 2 or higher. In this study, the correlation between histopathologic grade and magnifying colonoscopic findings ($r^2 = 0.807$) was better than that for histopathologic grade versus conventional colonoscopy ($r^2 = 0.665$). This study found that patients in whom MDE was observed during clinical remission frequently had a relapse within short periods (6 mo) compared with patients without these findings, and 50% of patients who underwent clinical remission still had active inflamed mucosa with MDE, which correlates with the results of previous studies in which 30% to 60% of patients in remission, as determined by clinical symptoms, were still in the active stage of ulcerative colitis based on histopathologic findings[49,62]. In our study we found that magnifying colonoscopy (MCS) grade was associated with the degree of histological inflammation in quiescent patients with ulcerative colitis, and might predict the probability of subsequent disease relapse in patients with ulcerative colitis in remission. Magnifying colonoscopy was performed in 112 patients with ulcerative colitis in remission. The relationship between pit patterns and histological disease activity was evaluated. Pit patterns in the rectal mucosa were classified into three MCS grades on the basis of size, shape, and arrangement (Figure 2). The patients were followed until relapse or a maximum of 12 mo. A positive correlation was identified between MCS grade and histological grade (Figure 3). Multivariate proportional hazard model analysis showed that MCS grade was a significant predictor of relapse. Kaplan-Meier estimate of relapse during 12 mo' follow up was found to increase with increasing MCS grade, with a percentage of 0 for grade 1, 19% for grade 2, and 43% for grade 3 (Figure 4). Although MCS grade positively correlated with histological grade, histological grade was less accurate predictors of disease relapse. One reason may be that they are assessed in biopsy specimens derived from a specific and limited area, whereas magnifying colonoscopy allows the observation of a more extended and representative area of colorectal mucosa, and accordingly greater accuracy by MCS grading.

CONCLUSION

Endoscopic parameters in patients with severe or refractory

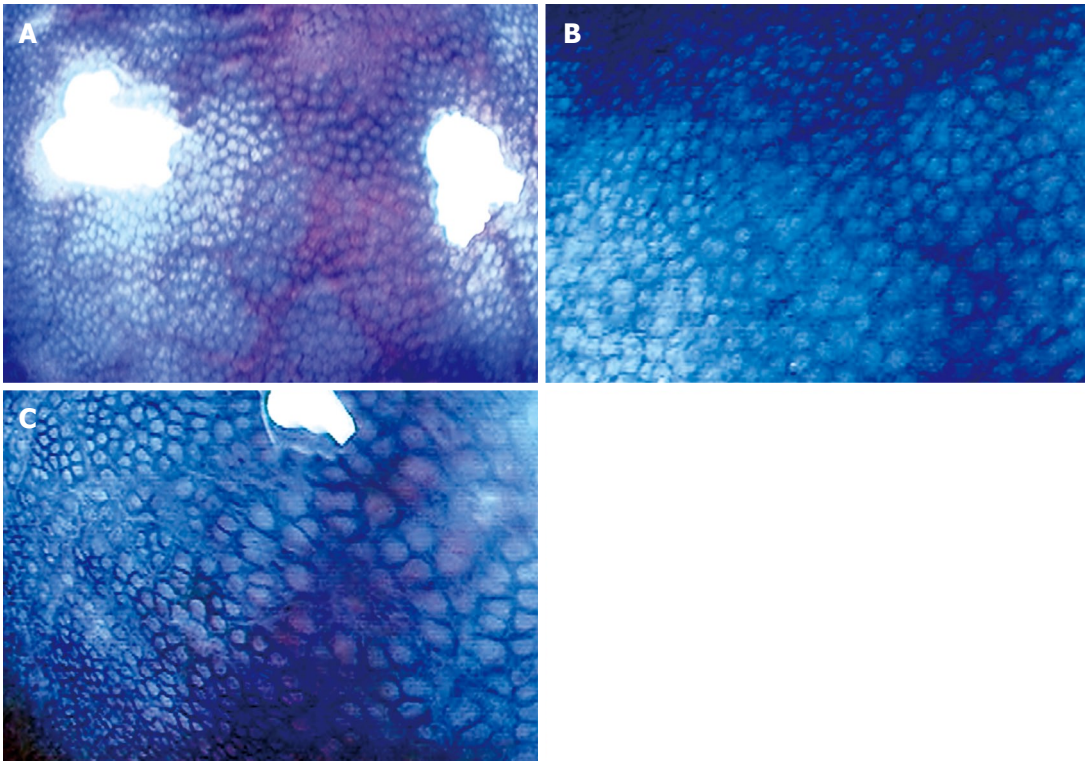

Figure 2 Grading of pit structures in the colorectal mucosa of patients with inactive UC. **A**: MCS grade 1, pits small, round, and regularly arranged; **B**: MCS grade 2, pits rather large, oval, and somewhat irregular in arrangement; **C**: MCS grade 3, pits of various shapes and sizes, and irregularly arranged.

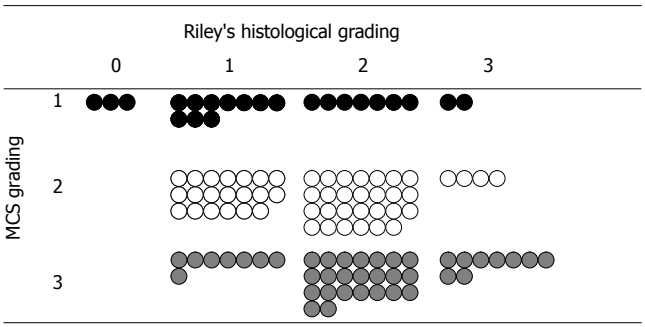

Figure 3 Correlation between grading of pit structures and histological findings ($P < 0.001$, Spearman's rank test). The number of colored ring indicates the number of the patients performed magnifying colonoscopy ($n = 112$).

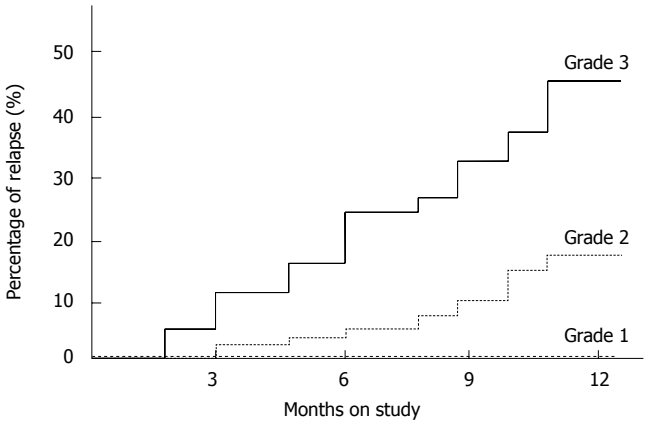

Figure 4 Kaplan-Meier survival analysis showing the cumulative proportion of patients who had a relapse according to MCS grade group.

UC may predict a clinical response of the therapies, such as cyclosporine or leukocyte removal therapy. Magnifying colonoscopy is useful for the evaluation of disease activity and for predicting relapse in patients with ulcerative colitis. Endoscopy is the cornerstone for diagnosis and evaluation of UC, and advanced imaging techniques, including chromoendoscopy, magnification endoscopy, confocal endomicroscopy, and spectroscopy, may aid in this field. Further studies remain to be done to define endoscopic predictors of response to therapy or outcome of UC patients.

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EDITORIAL

MUC1 and colorectal cancer pathophysiology considerations

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Abstract

Several lines of evidence point towards a biological role of mucin and particularly MUC1 in colorectal cancer. A positive correlation was described between mucin secretion, proliferation, invasiveness, metastasis and bad prognosis. But, the role of MUC1 in cancer progression is still controversial and somewhat confusing. While Mukherjee and colleagues developed MUC1-specific immune therapy in a CRC model, Lillehoj and co-investigators showed recently that MUC1 inhibits cell proliferation by a β-catenin-dependent mechanism. In carcinoma cells the polarization of MUC1 is lost and the protein is over expressed at high levels over the entire cell surface. A competitive interaction between MUC1 and E-cadherin, through β-catenin binding, disrupts E-cadherin-mediated cell-cell interactions at sites of MUC1 expression. In addition, the complex of MUC1-β-catenin enters the nucleus and activates T-cell factor/leukocyte enhancing factor 1 transcription factors and activates gene expression. This mechanism may be similar to that just described for DCC and UNC5H, which induced apoptosis when not engaged with their ligand netrin, but mediate signals for proliferation, differentiation or migration when ligand bound.

Key words: Mucin; MUC1; Glycoprotein; Colorectal cancer; Gastrointestinal oncology; Carcinogenesis; Metastasis; Tumorigenicity

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MUC1 is a structural membranous bound mucin, expressed on the apical borders of secretory epithelial cells which previously had many names such as DF3, episialin, CA5-3, PAS-O, polymorphic epithelial mucin (PEM), or epithelial membrane antigen[1].

MUC1 gene is located on chromosome 1q21 and has 1201 nucleotides. The N-terminal ectodomain of MUC1 (MUC1-N) consists of variable numbers of 20-amino-acid tandem repeats (VNTRs), with the number of repeats varying from 20 to 120 in different individuals[2]. These sites are subject to O-glycosylation that contributes to form a structure that extends beyond the glycocalyx of the cell. The protein has a protective function by binding to pathogens and also functions in a cell signaling capacity[3].

MUC1-N is tethered to the cell membrane as a heterodimer with the MUC1 C-terminal subunit (MUC1-C), which includes a 58-amino acid extracellular domain, a 28-amino acid transmembrane domain, and a 72-amino acid cytoplasmic tail that contains sites for tyrosine and serine phosphorylation[4]. Over expression, aberrant intracellular localization, and changes in glycosylation of this protein as found in most human carcinomas, confers anchorage-independent growth and tumorigenicity[5]. Other studies have demonstrated that over expression of MUC1 confers resistance to apoptosis induced by oxidative stress and anti-cancer agents[6]. Multiple alternatively spliced transcript variants that encode different isoforms of MUC1 have been reported, but the full-length nature of only some of them has been determined[7].

Several lines of evidence point towards a biological role of mucin and particularly MUC1 in colorectal cancer (CRC). A positive correlation was described between mucin secretion, proliferation, invasiveness, metastasis and bad prognosis[8-10]. When MUC1 was expressed at the deepest tumor invasive portion, lymphatic and venous invasion was more pronounced as well as lymph nodes and liver metastasis[11]. Correlation with bad prognosis was found in mismatch repair (MMR) - proficient colorectal tumors, but not in MLH1 negative tumors or in Lynch syndrome (HNPCC)[12].

But, the role of MUC1 in cancer progression is still controversial and somewhat confusing. While Mukherjee and colleagues, in a very sophisticated way, developed MUC1-specific immune therapy in a CRC model, Lillehoj and co-investigators showed recently that MUC1 inhibits cell proliferation by β-catenin-dependent mechanism[13,14]. A similar observation was described by Yuan and co-workers[15].

Interaction of the cytoplasmic tail of MUC1 with β-catenin has a significant effect on cell cycle and

proliferation. This process hardly happens in normal polarized epithelium, because MUC1 resides on the apical surface while β-catenin resides on the lateral surface. Loss of polarity during transformation creates a permissive environment for MUC1 and β-catenin interaction[7].

β-catenin can bind directly to the amino acid sequence 50-SAGNGGSSL-59 of the MUC1 cytoplasmic domain (a similar binding site is found on E-cadherin and APC proteins). The binding is promoted by phosphorylation of T41 by Ser/Thr kinase PKCζ and of Y46 by Src or EGFR[16]. Inhibition of β-catenin binding to MUC 1 is the result of phosphorylation of S44 by GSK3β that can also directly degrades β-catenin. Disruption of the β-catenin binding site in MUC1 suppresses its ability to induce anchorage-dependent and independent growth, indicating β-catenin binding to MUC1 is a critical component of its tumorigenc activity. MUC1 also protects β-catenin from degradation by GSK3β, and when co-localized with β-catenin in the nucleus co activates transcription of Wnt target genes[17-19]. MUC1 binding to β-catenin suppresses its ability to interact with E-cadherin at adherent junctions, leading to a breakdown in cell-cell interactions. GSK3β-mediated disruption of the complex restores the E-cadherin/β-catenin interaction[20].

In carcinoma cells the polarization of MUC1 is lost, and the protein is over expressed at high levels over the entire cell surface. A competitive interaction between MUC1 and E-cadherin, through β-catenin binding, disrupts E-cadherin-mediated cell-cell interactions at sites of MUC1 expression. In addition, the complex of MUC1-β-catenin enters the nucleus and activates T-cell factor/leukocyte enhancing factor 1 (Tcf/LEF-1) transcription factors and activates gene expression[18]. This enhances proliferation, and decreases cell-cell adhesion which may both increase carcinogenesis and metastasis. GSK3β interacts with the STDRSPYE motif in MUC1, phosphorylates the serine in this domain, and prevents binding of β-catenin[16].

It is proposed that APC binding prevents the formation of β-catenin-Tcf complex, and that MUC1 binding prevents β-catenin-α-catenin-E-cadherin complex. GSK3β interacts with β-catenin to restore β-catenin-E-cadherin complex, and with APC to bind β-catenin and prevent β-catenin-Tcf complex (Figure 1). The exact mechanism of MUC1 associated cancer cell proliferation and carcinogenesis is not well understood. MUC1 can bind β-catenin, prevents its entering the nucleus or activating Tcf/LEF-1, and inhibits proliferation. On the other hand, MUC1-C complex with β-catenin may enter the nucleus and the opposite action will take place. In both cases, β-catenin binding to MUC1 will prevent its binding to E-cadherin or to APC.

The cytoplasmic tail of MUC1 contains 4 tyrosine residues that are phosphorylated before binding β-catenin. It is speculated that MUC1 is a membranous receptor which maintains cell cycle progression or enhances apoptosis. Activating MUC1 will result in phosphorylation of the tyrosines on the cytoplasmic tail and binding β-catenin. This will prevent β-catenin from binding E-cadherin or activating Tcf/LEF-1 pathway. This mechanism may be similar to that just described for DCC and UNC5H, which induced apoptosis when not engaged with their ligand netrin, but mediate signals for proliferation, differentiation or migration when ligand bound[21].

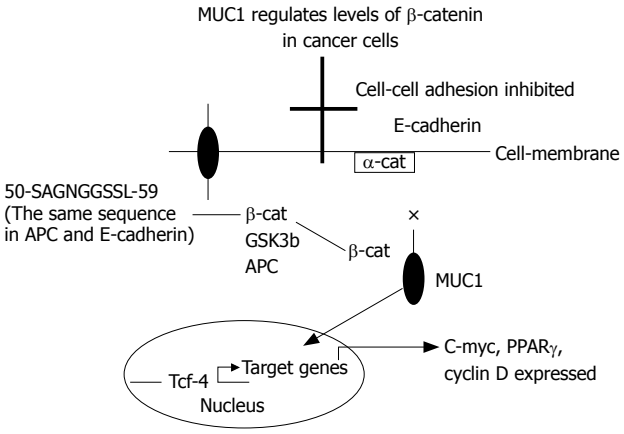

Figure 1 It is proposed that APC binding prevents the formation of β-catenin-Tcf complex, and that MUC1 binding prevents β-catenin-α-catenin-E-cadherin complex. GSK3β interacts with β-catenin to restore β-catenin-E-cadherin complex, and with APC to bind β-catenin and prevent β-catenin-Tcf complex. × = inhibition; arrow = enhancing.

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REVIEW

Necrotizing enterocolitis: A multifactorial disease with no cure

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Abstract

Necrotizing enterocolitis is an inflammatory bowel disease of neonates with significant morbidity and mortality in preterm infants. Due to the multifactorial nature of the disease and limitations in disease models, early diagnosis remains challenging and the pathogenesis elusive. Although preterm birth, hypoxic-ischemic events, formula feeding, and abnormal bacteria colonization are established risk factors, the role of genetics and vasoactive/inflammatory mediators is unclear. Consequently, treatments do not target the specific underlying disease processes and are symptomatic and surgically invasive. Breast-feeding is the most effective preventative measure. Recent advances in the prevention of necrotizing enterocolitis have focused on bioactive nutrients and trophic factors in human milk. Development of new disease models including the aspect of prematurity that consistently predisposes neonates to the disease with multiple risk factors will improve our understanding of the pathogenesis and lead to discovery of innovative therapeutics.

Key words: Necrotizing enterocolitis; Diagnosis; Pathogenesis; Prevention; Disease models; Vasoactive/inflammatory mediators

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INTRODUCTION

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease of neonates and remains one of the most common gastrointestinal emergencies in newborn infants[1]. Onset of NEC is often within the first three months of life and neonates who are of extremely low birth weight (< 1000 g) and under 28 wk gestation are the most susceptible[2]. Full term neonates account for 10% of all NEC cases while premature infants account for 90%[3]. With an incidence rate of 1%-5% for all newborns admitted to the NICU[1], a prevalence of 7%-14% of very low birth weight infants (VLBW, 500-1500 g)[4] and a mortality rate approaching 20%-50%[5], NEC continues to represent a significant clinical problem. In Canada, the incidence rate is 1.8 per 100 live births with a prevalence of 7% of VLBW infants[1]. Advances in obstetric and neonatal care have improved survival rates for smaller, more immature infants, and as more VLBW preterm infants survive the neonatal period, the population at risk for NEC increases[1]. No consistent association between sex, race, and rates of NEC has been identified. However, male VLBW infants and black infants are at greater risk of death[6]. Due to inadequate treatments and no effective preventative strategy, an estimated 20%-40% of babies with NEC require surgery[1] and 10%-30% experience significant morbidity including neurodevelopmental impairment, vision and hearing impairment, failure to thrive, feeding abnormalities, diarrhea, bowel obstruction, and short bowel syndrome[1,2,7]. The case fatality rate with surgical intervention is as high as 50%[1]. NEC is also a financial burden to the health care system with yearly hospital charges reported to be as high as $6.5 million in the US[8]. Thus, NEC continues to be an important health issue for preterm neonates.

DIAGNOSIS

Clinical signs and symptoms

The onset of NEC can occur suddenly within a few

Table 1 Bell's staging criteria for necrotizing enterocolitis

Stage	Systemic signs	Intestinal signs	Radiological signs
Ⅰ A (Suspected)	Temperature instability, apnea, bradycardia, lethargy	Poor feeding, emesis, ↑ pre-gavage residuals, mild abdominal distension	Normal or intestinal dilation, mild ileus
Ⅰ B (Suspected)	Same as above	Above and blood from rectum	Same as above
Ⅱ A (Proven)	Same as above	Above + absent bowel sounds + mild abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
Ⅱ B (Proven)	Above + metabolic acidosis + thrombocytopenia	Above + definite abdominal tenderness	Above + portal vein gas + possible ascites
Ⅲ A (Advanced)	Above + hypotension, respiratory acidosis, neutropenia	Above + peritonitis, marked distension of abdomen	Above + definite ascites
Ⅲ B (Advanced)	Same as above	Same as above	Above + pneumoperitoneum

hours or may be preceded by several days of feeding intolerance[7]. Age at presentation is inversely related to gestational age at birth, with full-term infants often presenting in the first few days of life[1]. NEC affects the gastrointestinal tract and, in severe cases, may have profound systemic impact[9]. Initial symptoms may be subtle and can include feeding intolerance (gastric residuals, bilious vomiting), bloody diarrhea, temperature instability, lethargy, apnea, bradycardia, decreased peripheral perfusion, delayed gastric emptying, ileus, abdominal distension, or tenderness and respiratory stress[1,9,10]. Non-specific laboratory abnormalities can include neutropenia, thrombocytopenia, hyponatriemia, hyperglycemia, metabolic acidosis, and bacteria or infectious products isolated from blood, urine, or stool[9,11]. Serial C-reactive protein could be useful in the management of the disease. C-reactive protein distinguishes stage I NEC from ileus or benign pneumatosis and high levels predict development of complications such as strictures, abscess, or need for surgery[12]. Because early signs of this disease are non-specific, sepsis may be suspected before NEC[1].

Pathological findings

The ileum and proximal colon are the most commonly affected sites in NEC although any segment of the gastrointestinal tract can be involved including the stomach[13]. Severity of bowel wall necrosis ranges from a small localized mucosal necrosis of a bowel segment to transmural necrosis of the entire small intestine and colon in most severe cases[7]. In more advanced stages of NEC, pathological findings include gastrointestinal bleeding, inflammation, bacterial overgrowth, intestinal distension with multiple dilated loops of small bowel, pneumatosis intestinalis and portal air, intestinal perforation, coagulative necrosis, hypotension, septic shock, pneumoperitoneum, and intraabdominal fluid[2,10]. In 1978, Bell and colleagues[14] proposed a system for the uniform clinical staging of infants with NEC. They classified infants as having stage Ⅰ (suspect), stage Ⅱ (definite), or stage Ⅲ (advanced)[14]. Bell's staging criteria for NEC are guidelines for the management of NEC (Table 1).

Ideally, nutrition intervention would begin when an infant has one or more risk factors for developing NEC (i.e. preterm birth) or is at an early stage of disease.

Diagnostic methods

Early diagnosis of gut ischemia and mucosal inflammation/necrosis is crucial in the prevention of NEC or the progression of the illness to late stages requiring surgery and/or bowel resection. An abdominal radiograph and a chest x-ray are used to diagnose gastrointestinal tract abnormalities and changes in the size and shape of the lung and heart, respectively[10,11]. The experimental and clinical methods for early detection of gut ischemia or NEC include serum hexosaminidase, plasma amylin, serum cytosolic β-glucosidase activity, plasma pro- and anti-inflammatory cytokines, serum creatinine kinase isoenzymes, cerebro-splanchnic oxygenation ratio, GI tonometry, rectosigmoid pH monitoring, urinary EGF, D-lactate, or thromboxane, breath hydrogen, and MRI[8]. Most of these methods do not have high clinical utility either due to accessibility issues, high costs, and need for expert assistance or due to their poor properties as a diagnostic/screening test especially in the early stages of NEC. Some infants present so acutely and severely that morbidity or mortality cannot be avoided despite best treatment. Identification of a biological marker for early disease should allow more timely diagnosis and treatment, but no ideal marker has yet been identified. The serum of symptomatic infants tends to contain high concentrations of certain cytokines such as interleukin-8 (IL-8)[15]. Some studies suggest that serum concentrations of fatty acid binding protein in the intestine and liver (I-FABP and L-FABP) could also be used as markers for NEC[16,17]. L-FABP concentrations at the onset of clinical signs are highest in infants later diagnosed with stage Ⅰ NEC and I-FABP concentrations are highest in infants who later develop stage Ⅲ NEC[16,17]. More sensitive and accurate imaging studies, such as ultrasonography, could become helpful adjuncts to abdominal films in the diagnosis of NEC[18]. Further research is needed on new approaches for the medical management of NEC that might prevent disease progression and improved surgical outcomes to reduce complications such as short bowel syndrome.

PATHOGENESIS

Although the exact etiology and pathogenesis of NEC remains elusive, it is well established that NEC is a

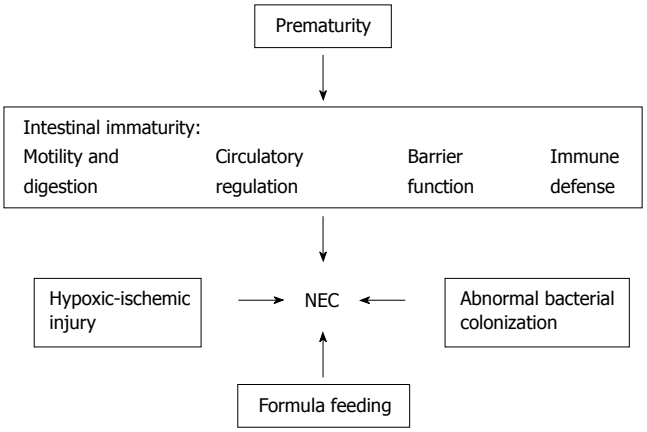

Figure 1 Pathophysiology of necrotizing enterocolitis (NEC).

complex, multi-factorial disease[2]. Besides pre-maturity, research suggests that other potential predisposing factors are hypoxic-ischemic injury, feeding with formula milk and colonization by pathological bacteria[1] (Figure 1).

Recent studies have shown that carrier state of genetic polymorphisms may be associated with perinatal morbidity, including NEC[19]. The hallmarks of NEC are loss of gastrointestinal motility, disruption of intestinal mucosal integrity, and mucosal inflammation, all of which result in the final common pathway, intestinal apoptosis and necrosis[4,20-23]. Several inflammatory and vasoactive mediators including platelet activating factor (PAF), cytokines, nitric oxide (NO), endothelin-1 (ET-1), prostaglandins, leukotrienes, and reactive oxygen species (ROS) are considered to play a synergistic and central role in the final inflammatory pathway leading to NEC[20]. The consequent breakdown of the mucosal barrier and impaired ability of the mucosa to heal leads to the self-perpetuating vicious cycle resulting in severe NEC, shock, sepsis, and sometimes death[8,24,25].

Prematurity

Prematurity is the only factor consistently found in epidemiological studies to be an independent determinant of NEC[2]. Up to 90% of infants with NEC are of low birth weight and the disease is more frequent and severe in those infants with the earliest post-conceptual age[7]. The increased susceptibility is attributed to an immature mucosal barrier and barrier response, changing intestinal microflora and increasing enteral volumes[2,23].

Immature intestinal motility, digestion, and barrier function

Intestinal motility is a critical factor in clearing antigens presented to the intestinal mucosal barrier from the gut lumen. The time available for absorption depends on the speed of luminal contents. Migratory motor complexes act as "house keepers" to propel luminal components caudally along the length of the small intestine. Immature intestinal motility and digestion may predispose preterm infants to NEC. Fetal studies in both animals and humans suggest that development of gastrointestinal motility begins in the second trimester, but matures in the third trimester[26-28]. Studies of intestinal motility have shown that premature infants can have immature motility patterns when compared with full-term infants and that maternal-fetal disease states that induce fetal hypoxia can further reduce postnatal intestinal motility[29-31]. Immature motility patterns alter normal peristaltic activity and result in overgrowth of anaerobic bacteria in the small intestine with malabsorption of dietary nutrients[23]. In addition, to impaired intestinal motility, premature infants have not yet developed the ability to digest and absorb nutrients and incompletely digested molecules could contribute to intestinal injury[32,33]. Lebenthal and Lee[34] showed that the function of the exocrine pancreas is limited in infants and that pancreatic insufficiency may last through the first year of life. Lack of stimulation of gastric acid and pancreaticobiliary secretions and their resulting proteolysis may adversely affect the intestine by allowing a greater bacterial and/or antigenic load. Thus, impaired digestion of nutrients, coupled with delayed transit time and bacterial overgrowth could result in intestinal injury with immature host and barrier defenses.

If structural or biochemical components of the intestinal epithelial barrier are not fully developed, bacteria may gain access to deeper tissues and cause inflammation. Intestinal epithelia are joined by tight junctions that regulate intestinal permeability and form by 10 wk gestation[35]. Studies show that intestinal permeability to macromolecules including immunoglobulins, proteins, and carbohydrates is highest in premature infants, particularly in those diagnosed with NEC[20,23]. When fully developed, the intestinal epithelial barrier can allow selective permeability to small ions, absorption of nutrients and control of bi-directional fluid flow. Enterocytes use chloride ions and water secretion to flush unwanted pathogens or toxins from the intestinal lumen. Fetal intestinal secretion and absorption are underdeveloped in preterm infants and mature gradually, under the influence of amniotic fluid, from 26 wk gestation to full-term[32]. Therefore, pathogens or toxins might not be efficiently washed from the intestinal lumen and could translocate across the leaky intestinal barrier in preterm infants.

Goblet cells are found throughout the small and large intestine. These specialized enterocytes secrete mucins, forming a thick protective layer over the intestinal mucosa. This mucus layer impedes direct microbial-epithelial binding and enhances removal of adherent bacteria[36]. Preterm infants have immature goblet cells. Developmental expression of mucin genes changes throughout the intestine and matches adult pattern expression between 23 and 27 wk gestation[37]. Microvilli of immature intestine also have altered glycosylation patterns[38]. Since carbohydrate sequences are recognition and attachment sites for microbes, changes in glycosylation patterns may influence the bacterial colonization pattern of the gut. An immature mucin layer might lead to increased intestinal permeability and enhanced bacterial adherence, potentially breaching the intestinal epithelial barrier and increasing susceptibility to injury.

Another aspect of the intestinal epithelial barrier that may not be functioning correctly in preterm infants is biochemical defenses. Paneth cells, which are specialized

secretory enterocytes located at the base of small intestinal crypts, secrete lysozyme, phospholipase A_2, and antimicrobial peptides (also secreted by absorptive enterocytes) that regulate composition and distribution of different bacterial populations[39,40]. Defensins (α and β) and cathelicidins are the two main families of antimicrobial peptides produced by intestinal cells[40]. These antimicrobial peptides have bioactivity against a wide range of microbes including bacteria, viruses, and fungi[41]. Some have a pro-inflammatory role and chloride secretory activity[42,43]. A better understanding of how biochemical defense molecules modulate host immune defenses *in vivo* should contribute to understanding the pathophysiology of NEC.

It is well established that growth factors, growth factor receptors, or their related signal transduction pathways are aberrant in the immature intestine. Epidermal growth factor (EGF) is a major trophic factor for the development of the intestine and the EGF receptor has been identified on the basolateral surface of enterocytes[44]. Exogenous infusion of EGF *in utero* has been shown to accelerate the maturation of intestinal enzyme activity as well as stimulate intestinal growth[45]. In the amniotic fluid, there is an increasing concentration of EGF as gestation progresses[46]. In fact, the salivary level of EGF is directly proportional to the gestational age of the infant[46]. Moreover, expression of EGF receptor involved in intestinal maturation and restitution is decreased in the preterm infant[7]. Recently, human data suggests a link between EGF production and NEC. Serum and salivary levels of EGF are significantly reduced in infants with surgical NEC[47]. Preliminary studies on the clinical use of EGF report improved epithelial regeneration with no significant toxicities[48].

It is unclear whether the intestinal epithelium of the infant can respond to injury to the same extent as the adult. In animals, infant intestinal epithelium turnover is much slower (4-5 d) than the adult (2 d)[49]. If the same finding holds true in humans, regeneration of injured mucosa in the infant will be much slower than the adult. Trefoil factor peptides (TFF1-3) are part of the protective mechanism operating in the intestinal mucosa and play a fundamental role in epithelial protection, repair, and restitution[50]. These secreted peptides have been identified in a site-specific pattern in the gastrointestinal mucosa and their expression has been shown to be up-regulated in early stages of mucosal repair[51,52]. The role of trefoil peptides in neonatal mucosal protection has not been well investigated. Intestinal trefoil factor is developmentally regulated and deficient in the premature neonate[20]. Recent studies demonstrated a lack of trefoil factor expression in response to NEC in the premature gut[53] and an insufficient proliferative response to reverse the mucosal insult observed in NEC[54]. Thus, impaired restitution of the mucosa may contribute to the cascade of bowel necrosis and generalized sepsis characteristic of NEC.

Immature intestinal immunity

Although the fetus at term may be sensitized to certain antigens, the fetus does lack a fully functional immune system and has a sterile gastrointestinal tract. Changes occur at, and soon after birth, in order that the immune system of the neonate becomes competent and functional and that the gut becomes colonized with bacteria. Exposure to bacteria during birth and from the mother's skin and the provision of immunological factors in breast milk are amongst the key events that promote maturation of the infant's gut and gut-associated immune system[55]. Dendritic cells play an important role in the initiation of the immune response. Microbial and antigenic-priming of dendritic cells develops different signals that drive the differentiation of naïve Th cells into Th1, Th2 or T regulatory cells[56]. Developmental changes in glycosylation patterns of immature dendritic cells may play an important role in development, maturation, and immune regulation[57].

Innate and adaptive immune defense systems are abnormal in developing neonates[20]. A possible mechanism for the pathophysiology of NEC is that reduced inflammatory signaling could allow bacterial overgrowth. Newborns are Th2 polarized and do not respond efficiently to IFN-γ[58]. Moreover, newborn macrophages exposed to LPS are defective in producing pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-12 (IL-12)[58,59]. Interestingly, inhibitory activity to toll receptors in neonatal but not adult plasma has been detected[60]. Neonatal monocyte and T cell production of the anti-inflammatory cytokines interleukin-10 (IL-10) and TGF-β are developmentally delayed[61]. Preterm infant polymorphonuclear (PMN) counts are lower and premature neonatal macrophages have reduced respiratory burst activation as compared with term newborns[62]. Under conditions of stress, PMNs of term and preterm infants do not function with normal phagocytic and microbicidal activities[63]. PMNs isolated from the blood of term and preterm neonates consistently display diminished chemotactic and adhesion capacities[64]. It is known that intestinal lymphocytes are decreased in neonates (B and T cells) and do not approach adult levels until 3-4 wk of life[20]. Newborns also have markedly reduced synthesis of secretory IgA and IgG in response to mitogens, reflecting decreased activity in the intestine[20]. Failure to activate inflammatory pathways in premature infants might prevent induction of anti-apoptotic, cytoprotective factors. Thus, developmental immaturity of the inflammatory response could increase susceptibility to apoptosis when cells are challenged by environmental stress.

Long-term survival requires inflammation as a defense mechanism, however, uncontrolled inflammation results in intestinal barrier damage, translocation of pathogens, further inflammation, and tissue damage. Some *in vitro* studies suggest that immature intestinal cells have a propensity for exaggerated inflammatory responses to pathogenic stimuli and researchers postulate that developmentally deficient expression of the NF-κB inhibitor IκB might allow greater NF-κβ activity[65,66]. NF-κB is a nuclear transcription factor that enhances the production of inflammatory mediators and is essential for innate immunity, adaptive immunity and cell survival[67]. In the human newborn, PAF-AH activity is decreased and

Table 2 Ischemic events associated with necrotizing enterocolitis

Perinatal asphyxia
Polycythaemia
Cyanotic congenital heart disease
Patent ductus arteriosis
Medications that ↓ superior mesenteric blood flow (cocaine)
Maternal pre-eclampsia

PAF synthesis pathways are increased. This imbalance places the newborn at special risk of an increased PAF response before adequate immune stimuli are developed[7].

Hypoxia-ischemia

Pathological findings of NEC associated with ischemic events (coagulative necrosis, Table 2) and the fact that NEC most commonly occurs in the distal ileum and proximal colon, which make up the watershed area of the superior and inferior mesenteric arteries, suggests that derangement of the circulatory system is involved[7].

Preterm neonates are more susceptible to hypoxia and intestinal ischemia because their system for regulating vascular resistance is poorly developed[68]. The most distinguishing feature of the newborn intestinal circulation is its very low vascular resistance due to substantial generation of endothelial derived NO when compared with ET-1[69]. Immature intestine handles the increased metabolic demands of growth by increasing blood flow and oxygen consumption[10]. However, during episodes of cardiovascular stress, infants are less able to raise intestinal blood flow and metabolic demands overwhelm the infant's ability to increase oxygen consumption[10]. Defective pressure flow autoregulation in response to hypotension occurs[68]. Consequently, hypoxia in tissues can occur. Hypoxia increases production of vasoconstrictor ET-1 and ischemia/reperfusion compromises production of endothelial derived vasodilator NO[69]. Thus, an imbalance between ET-1 and NO production by the newborn intestine following an initial ischemic insult might exacerbate existing intestinal ischemia. Whether the hypoxic/ischemic insult is primary, secondary or both an initiating factor and end result remains controversial. One plausible mechanism that is often cited is the "diving reflex", whereby blood flow is preferentially diverted to the heart and brain in preference to less vital organs[1]. Very early descriptions regarding the pathogenesis of NEC suggested a primary or early role for ischemia and hypothesized that a hypoxic/ischemic insult directly damaged the intestinal mucosa disrupting the neonatal gut barrier and promoting bacteria translocation and the inflammatory cascade[21]. Animal models suggest that NEC may not occur without significant reperfusion injury resulting from the generation of oxygen-free radicals at the restoration of blood flow and oxygen delivery after ischemia[9]. Inflammatory mediators may also cause intestinal ischemia by up-regulating ET-1 production and the expression of its receptor ET_A[69]. Current studies show a stronger association with prematurity, rapid feeding, abnormal intestinal colonization and inflammatory mediators than with ischemia[23]. Hypoxia-ischemia might contribute to the pathogenesis of NEC, but it likely plays more of a secondary role.

Formula feeding

Enteral feeds have a firm association with NEC as 90%-95% of NEC cases occur in infants with initiation/re-initiation of enteral feeds or recent volume advancement[2,20]. Infants receiving hyperosmolar formulas or rapid volume advancements are at greatest risk[20]. Although the mechanism is not well understood, enteral feeding has been reported to contribute to the development of NEC through disruption of mucosal integrity, blood flow and motility and through provision of a bacterial substrate[2,10]. Raising milk intake increases metabolic demands, making it difficult for the infant to expand mesenteric blood flow to meet demands[10]. As a result, intestinal hypoxemia may occur. Increased proliferation of potentially pathogenic bacteria may go on to invade the bowel wall[10]. Although the newborn gastrointestinal tract is sterile at birth, bacterial colonization occurs within hours[10]. Contact with the mother's vaginal flora begins this process, which is further developed by oral feedings and exposure to the environment[10]. In fact, breast fed infants are 10X less likely to develop NEC than formula fed infants, suggesting that breast milk contains multiple bioactive factors that influence host immunity, inflammation and mucosal protection. Breast milk notably increases the diversity of gastrointestinal bacterial colonization and contains immunomodulatory factors such as secretory immunoglobulin A, leukocytes, mucin, lysozyme, cytokines, lactoferrin, growth factors, enzymes, oligosaccharides, and polyunsaturated fatty acids not provided in commercially available neonatal formula preparations[20,55]. These factors are capable of inducing mucosal protection and neutralizing potent pro-inflammatory cytokines and phospholipids[55]. Glutamine and nucleotides may help in gastrointestinal cell metabolism[10]. EGF can directly improve gastrointestinal function and promote gut maturity[25].

Abnormal bacterial colonization and infection

The well-documented epidemics of NEC and the improvement in incidence and severity following the implementation of strict infection control measures validates the role of infection in the pathogenesis of NEC[2]. Furthermore, the regions of the intestine that are most often associated with NEC (ileum and proximal colon) have very high bacterial loads. Moreover, no cases of NEC have been described *in utero*, supporting the importance of bacteria colonization in the pathophysiology of NEC[20].

Although several bacterial and viral species have been associated with outbreaks of NEC (*Clostridium* sp, *Klebsiella* sp, *Staphylococcus epidermis*, *Escherichia coli, Rotavirus*), no single pathogen has been identified as causative and the ability of the microflora to colonize the epithelium and to ferment unabsorbed nutrients may be more important than the strain itself[13,70]. Recently, early abnormal colonization of stools with *Clostridium perfringens* has been correlated with later development of NEC[71]. *Clostridium perfringens* has

been isolated from 40% of infants with NEC, compared with 13% of controls[71]. Premature infants are especially susceptible to intestinal colonization by pathological bacteria due to their daily exposure to nosocomial flora and the likelihood of exposure to antibiotics and steroids on admission to NICUs[72].

Colonization of the gastrointestinal tract of the premature infant differs greatly from that of the healthy term infant[9,20]. Patterns of intestinal colonization also vary according to the type of enteral feeding[3]. The colonization of the hospitalized, premature infant gastrointestinal tract has less species diversity and fewer anaerobic species of *Lactobacillus* and *Bifidobacterium*[9,20]. Breast-fed infants have large amounts of protective, gram-positive *Bifidobacteria* in their intestine, contrasting with formula-fed neonates who are colonized predominantly by potentially pathogenic gram-negative *Enterobacteria*[3]. Gram-positive bacteria yield lactic acid during carbohydrate metabolism, which is readily absorbed from the intestinal lumen, whereas gram negative-bacteria ferment lactose into hydrogen, carbon dioxide and organic acids, producing distension, increased intraluminal pressure, decreased mucosal blood flow and pneumatosis intestinalis[3]. Enteral feeds and poor gastrointestinal motility associated with immaturity may promote stasis and bacterial overgrowth[3]. This microbial imbalance may represent a fertile environment for the pathologic overgrowth, binding and invasiveness of otherwise non-pathogenic intestinal bacterial species capable of triggering the inflammatory cascade with resultant NEC[9]. Recently, inappropriate immunologic responses of premature enterocytes to bacteria colonization have been implicated in the development of NEC[13]. Reports indicate that pathogenic stimuli including *Salmonella* and *E. coli*, produce exaggerated pro-inflammatory responses in immature intestinal epithelial cells[65,66].

Abnormal expression of pattern recognition receptors that recognize microbial signatures might also affect the way in which the intestine in premature infants responds to bacterial colonization. One of the first pro-inflammatory molecules to cross the intestinal barrier is lipopolysaccharide (LPS), which is a principal component of the outer cell wall of Gram-negative bacteria that recognizes and binds to toll like receptor 4 (TLR4)[21]. Circulating LPS is increased in patients with NEC, which inhibits epithelial restitution and initiates inflammatory signaling cascades within the enterocyte including activation of transcription factor NF-κB and expression of enzymes that produce apoptotic NO and pro-inflammatory eicosanoids and cytokines[21] (Figure 2).

In rats, intestinal epithelial cells up-regulate expression of TLR4 in response to stress-induced production of PAF, suggesting that up-regulation of TLR4 might explain how NEC develops in this animal model[73]. It remains unclear whether bacterial translocation into submucosa is a prerequisite for disease or whether the activation of the Toll-like receptors from endotoxin and other bacterial cell wall products is adequate to initiate the final common pathway of intestinal injury[20]. For premature infants at risk for NEC, there may be increased passage of bacteria

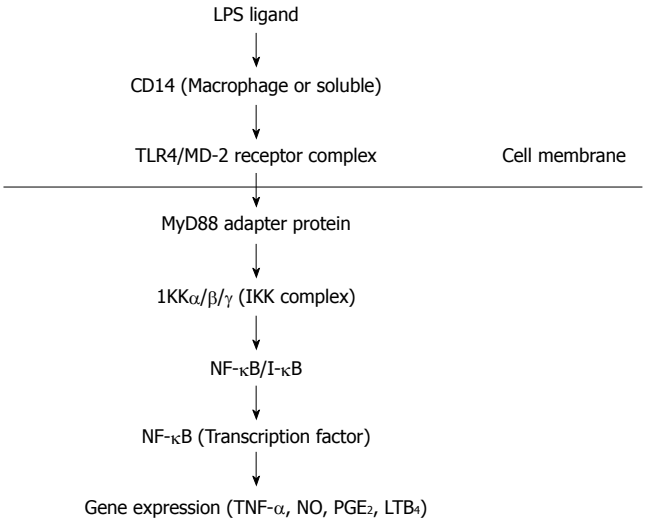

Figure 2 LPS-Induced signaling pathways leading to NF-κB activation.

from the gut into the systemic circulation and exaggerated pro-inflammatory responses[10]. Most of the defenses that would normally prevent passage of bacteria across the mucosal barrier-a well-functioning immune system, intact mechanical defenses and normal intestinal microflora are impaired in patients who are at risk for NEC[10]. Gram-negative bacteria translocate to regional lymph nodes and activate resident macrophages to release inflammatory mediators[2]. Bacteria endotoxins can leak into the systemic circulation causing release of inflammatory mediators, intestinal damage, shock and death[2,10].

Commensal bacteria interact symbiotically with the mammalian intestine to regulate the expression of genes important for barrier function, digestion, and angiogenesis[74]. Commensal bacteria can inhibit inflammatory pathways and perhaps contribute to the maintenance of homeostasis[75]. *In vitro* experiments show that a wide range of commensal bacteria can reduce inflammatory signaling in intestinal epithelia by inhibition of the NF-κB signaling pathway[76,77]. Preliminary work suggests that early colonization by probiotics (facultative anaerobes such as *Lactobacilli* and *Bifidobacteria*) reduces the risk of NEC in very low birth weight infants[78,79].

Genetics

Investigation of factors that might cause a genetic predisposition for NEC might eventually allow specific treatments or preventative strategies for the infants most at risk for this disease. Current technology allows for the detection and evaluation of genetic polymorphisms and their influence on disease development. Studies are now emerging which investigate the potential importance of specific polymorphisms for known NEC-associated inflammatory mediators. The presence of genetic variance may contribute to the inter-individual variance of cytokine response to inflammatory stimuli[19]. A family of intracytoplasmic pathogen recognition receptors has been shown to sense invading bacteria and activate gene transcription pathways that regulate immune and inflammatory responses. In a recent clinical study, VLBW

infants with mutations in a member of this family, NOD2, demonstrated increased susceptibility to bacterial sepsis[80]. Genetic polymorphisms of CD14, TLR4, and NOD2 are not associated with NEC in VLBW infants[81]. In preliminary studies, VLBWI with NEC were shown to be less likely to possess the interleukin-4 (IL-4) receptor α-chain mutant allele compared to infants without NEC[19]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced Th2 state[19]. It is speculated that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC[19]. The risk of NEC has also associated with the frequency of the IL-18^{607} AA genotype. The frequency of the AA genotype is significantly higher in infants with stage 3 NEC compared to stages 1 and 2[19]. Thus, the presence of the AA genotype may adversely affect the outcome of NEC through altered IL-18 levels, a cytokine that induces IFN-γ and amplifies Th1 cytokine production and IL-8 accumulation[19]. Another possible genetic factor is the pro-inflammatory cytokine TNF-α. In animal models, pretreatment with anti-TNF-α reduces the incidence and severity of NEC[82,83]. Investigators have not reported a genetic link between TNF-α gene variants and the disease[84].

Vasoactive and inflammatory mediators

Bacterial colonization and enteral feeds coupled with damage to and loss of the integrity of the immature gastrointestinal mucosa trigger the final common pathway leading to the development of NEC[9]. Inflammatory mediators are responsible for protecting the body from invading organisms and play a vital role in the pathogenesis of NEC[3]. Inflammation can be initiated by a variety of factors including exposure to the bacterial cell wall product, endotoxin, and ischemia reperfusion[20]. The release of potent biologically active phospholipids, cytokines, products of arachidonic acid metabolism, vasoactive mediators, neurotransmitters, and reactive oxygen species from the immature and damaged gastrointestinal cells and inflammatory cells amplify the inflammatory response, leading to tissue damage and NEC[9]. Studies of animals and human cell lines suggest that the balance between pro-inflammatory and anti-inflammatory modulatory factors in premature infants is pro-inflammatory[9].

NO

NO is a short-lived, labile free radical gas that reacts with a variety of biologically active substances[85]. Such reactions result in both local and systemic effects that modulate the inflammatory response in a variety of tissues[21]. The synthesis of NO in biological systems is regulated by nitric oxide synthase (NOS), which catalyzes the oxidation of the amino acid L-arginine to release citrulline and nitric oxide[21]. Although diverse molecular reactions of NO have been identified in physiological and pathological systems, the fastest and most biologically relevant reaction of NO is with superoxide to produce the potent oxidant peroxynitrite[21]. Peroxynitrite is a key intermediate that is generated at inflammatory sites and is responsible for mediating tissue injury, in part, through lipid peroxidation[21]. Three isoforms of NOS exist: Neuronal (nNOS) and endothelial (eNOS), which are calcium/calmodulin dependent and constitutively expressed, releasing physiologically low concentrations of nitric oxide (pM) and the calcium independent inducible isoform (iNOS), which releases toxic concentrations of nitric oxide (nM) in response to infection and inflammatory stimuli[86]. All three isoforms are expressed in the gastrointestinal tract[21,86]. The constitutive forms are expressed by endothelial cells, enteric neurons, gastric epithelial cells, and enterocytes[86]. In the gastrointestinal tract, NO mediates inhibitory nerve-related relaxation of intestinal smooth muscle and plays a role in regulating gut mucosal blood flow, mucosal permeability, intestinal motility and mucosal protection[85,86]. Normal smooth muscle sphincteric function as well as coordinated peristalsis is dependent on the integrity of intrinsic nitric oxide neurons of the myenteric and submucosal networks throughout all regions of the gut wall[85]. NO also maintains intestinal microvascular integrity by inhibiting platelet aggregation and leukocyte adhesion[86]. Ontogenic variation in constitutive NOS activity has been observed in different animal species, in humans and in different organs[85]. By contrast, iNOS expression and activity within the intestinal epithelium is normally low, although it may be increased 15-fold after 4 h stimulation with LPS[21]. NO and peroxynitrite have anti-microbial properties and play important roles in host defense against pathogens[86]. However, sustained high levels of NO production promote bacteria translocation following insults such as endotoxemia and ischemia-reperfusion injury[86]. The induction of iNOS mRNA expression by inflammatory mediators has been seen in animal models of NEC and in intestinal resections from patients with NEC where the predominant source of iNOS activity was the enterocytes. Endothelial NOS function is compromised in human intestine resected for NEC[87]. Poorly coordinated production of NO by NOS isoforms occur during the early phase of the disease and are involved in altered intestinal blood flow, ischemic damage, disassembly of tight junction proteins, and impaired healing typically seen in NEC[13]. Research suggests that NO participates in the pathogenesis of NEC by directly damaging the enterocyte monolayer and by disrupting the ability of the mucosa to repair itself[21]. Extensive apoptosis has been shown in the enterocytes of the apical villi of infants with NEC and this correlates with the degree of nitrotyrosine immuno-staining, a marker of NO release and tissue reactivity[88]. Toxic concentrations of NO have also been shown to decrease enterocyte proliferation and inhibit enterocyte migration[21]. It is proposed that peroxynitrite interferes with EGF receptor signaling in enterocytes[89].

ET-1

ET-1, a potent vasoconstrictor agent, is produced at several sites within the intestine including vascular endothelial cells, submucosal stroma, and circularis muscularis layers of the gut wall[69]. Although constitutively produced,

ET-1 production is increased by a wide range of stimuli including reduced flow rate, hypoxia and inflammatory cytokines[69]. ET-1 generates a profound degree of ischemia that is sustained for hours because of a unique interaction between ET-1 and its receptor[69]. If not balanced by concomitant vasodilatory stimuli, ET-1-induced ischemia can generate hypoxia and tissue death[69]. ET-1 induces vasoconstriction by binding to ET_A receptors present within the newborn intestine and whose activation can generate intestinal tissue damage when excessive amounts of ET-1 are present[69]. Recently, ET-1 was demonstrated to be associated with NEC. It has recently been shown that the tissue concentration of ET-1 is greater in human preterm intestine that demonstrates histologic evidence of NEC[90]. Moreover, it has been demonstrated that arterioles harvested from intestine exhibiting histologic evidence of NEC exhibits vasoconstriction and that the vasoconstriction can be reversed by blocking ET_A receptors[90].

Serotonin

Serotonin is an intermediate product of tryptophan metabolism and is primarily synthesized and released by enterochromaffin cells of the intestine (90%) and enteric/brain neurons (10%) in response to calcium influx, physical mucosal stimulation, nutrients, hypoxia, and elevations in intraluminal pressure[91]. Levels of serotonin in the gastrointestinal tract are regulated by a serotonin uptake transporter, SERT, present in the mucosa and enteric nerves[92]. The major function of serotonin in the gastrointestinal tract is stimulation of bowel motility, epithelial secretion, and vasoconstriction through serotonin receptor binding[91]. Disruption of serotonin homeostasis and signaling is commonly seen in several gastrointestinal motility and inflammatory disorders including bowel obstruction and inflammatory bowel disease[93]. In inflammatory bowel disease, serotonin levels and enterochromaffin cell numbers are increased[93]. The inflamed intestinal tissue releases more serotonin, has a reduced capacity to remove serotonin and the serotonin receptors are desensitized[93]. Some cases of NEC have been associated with maternal use of paroxetine, a long-acting serotonin re-uptake inhibitor[94].

PAF

PAF, an endogenous phospholipid with powerful pro-inflammatory actions, is synthesized by neutrophils, macrophages, endothelial cells, and enterocytes in response to endotoxin and hypoxia[10]. PAF formation begins with the conversion of a phosphatidylcholine precursor to a biologically inactive intermediate, lysoPAF, under the influence of cytosolic phospholipase A_2[73]. Subsequent acetylation of lysoPAF at the n-2 position by acetyltransferase completes PAF synthesis[73]. PAF has a very short half life as it is rapidly degraded by PAF-acetylhydrolase[73]. In the human newborn, PAF synthesis pathways are increased and the activity of the PAF-degrading enzyme PAF-acetylhydrolase is decreased[7]. This imbalance places the newborn at special risk of an elevated PAF response before adequate immune stimuli are developed[7]. Formula does not contain PAF-AH like human milk, leaving susceptible neonates at greater risk for NEC. PAF exerts its effects by binding to PAF receptors present on most cells[73]. Interestingly, PAF receptors are most highly concentrated in the ileum, the region of the intestine where NEC is very prominent[24]. Down-stream signaling includes elevation of cytoplasmic free calcium and stimulation of protein kinase C, mitogen-activated protein kinase (MAPK), and NF-κB with production of inflammatory molecules including iNOS, TNF-α, ET-1, IL-1, IL-6, and IL-8[73]. PAF also activates pathways that result in caspase activation and apoptosis[73]. PAF is one of the most extensively studied mediators of intestinal injury and has been indicated as an important mediator in several animal models and human analyses of NEC[4]. PAF infusion causes intestinal necrosis in animals and PAF receptor antagonists prevent injury following hypoxia, endotoxin challenge and ischemia reperfusion injury[20]. Human patients with NEC show high levels of PAF and decreased levels of plasma PAF-AH with levels correlating with NEC severity[4,24]. In immature or mildly damaged mucosa, the close proximity of bacteria and intestinal epithelial cells aids transcellular permeation of PAF into the mucosa and local entry of bacteria[10]. Injection of LPS and bacterial invasion leads to increased production of platelet activating factor, release of secondary inflammatory mediators and further mesenteric ischemia and damage causing clinical NEC[10,24].

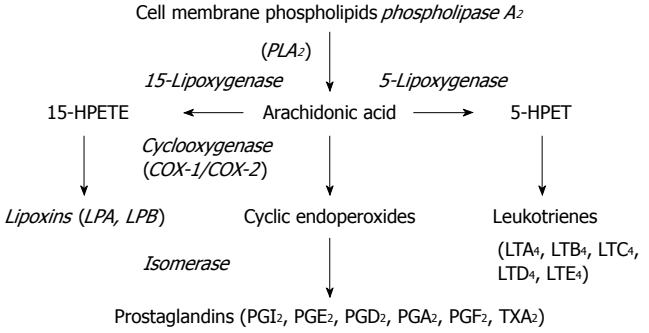

Figure 3 Metabolic pathways of arachidonic acid and eicosanoid production.

Eicosanoids

Arachidonic acid is a polyunsaturated fatty acid that is liberated from cell membrane phospholipids and serves as a precursor for many immune active lipids, collectively called eicosanoids (oxygenated C20 fatty acids)[95,96]. Classes of eicosanoids that signal in the immune system include prostaglandins, leukotrienes and lipoxins[95]. The major producers of eicosanoids are platelets, monocytes, macrophages, neutrophils, and mast cells, although with the exception of leukotrienes, they are also synthesized by a variety of non-immune cell types[95]. These lipid mediators are not stored in cells rather they are synthesized from arachidonic acid via three major metabolic pathways, either constitutively or in response to cell-specific trauma, stimuli, or signaling molecules[96](Figure 3).

The 15-lipoxygenase metabolic pathway results in the production of 15-hydroxyperoxy-eicosatetraenoic acid (15-HPETE) that serves as a precursor for the lipoxins

Table 3 Eicosanoid synthesis and actions

Eicosanoid	Cell/tissue origin	Target cell/tissue	Receptor	Action
PGE2	Most cells	Many cells	EP1-EP4	Fever, pain
PGI2	Endothelium	Platelet VSMC	IP	Declumping, vasodilation
PGF2	Uterus	Uterine SMC	FP	Contraction
PGD2	Mast cells	Lung Th2 cells	DP1/DP2	Asthma, chemotaxis
TXA2	Platelets	Platelet VSMC	TPα/TPβ	Aggregation, vasoconstriction
LTB4	Macrophage monocytes	Neutrophils	BLT1/BLT2	Promotes chemotaxis
LTC4/LTD4/LTE4	Macrophage monocytes	Lung SMC	BLT3/BLT4	Bronchoconstriction
LXA4	Leukocytes	Neutrophil	LXA4 R	Inhibits chemotaxis
LXB4	Leukocytes	NK cells	?	Inhibits cytotoxicity

LPA and LPB. Lipoxins exert anti-inflammatory activities through stimulation of macrophage phagocytosis of apoptotic neutrophils and inhibition of natural killer (NK) cell cytotoxicity and pro-inflammatory factor production[97,98].

Prostaglandins are end products of metabolism of arachidonic acid by constitutive and inducible cyclooxygenase isoforms (COX-1 and COX-2, respectively)[96]. The COX-1 enzyme accounts for basal prostaglandin synthesis for homeostatic regulation while COX-2 is involved in the synthesis of pro-inflammatory prostaglandins[96]. Leukotrienes are generated during the metabolism of arachidonic acid by the 5-lipoxygenase pathway and exert pro-inflammatory effects[95]. Prostaglandins and leukotrienes are emitted from their cell of origin and exert their effects in an autocrine or paracrine fashion by signaling through specific G-protein coupled receptors[95] (Table 3).

Cytokines

Pro-inflammatory cytokines are multifunctional proteins produced in response to inflammatory stimuli that communicate to the surrounding tissue the presence of infection or injury. Several pro-inflammatory cytokines that mediate inflammatory cell recruitment through activation and amplification of the immune response in local host defense have been implicated in NEC including TNF-α, IL-1β, IL-6, IL-8, IL-12, and IL-18[99-101]. Anti-inflammatory cytokines modulate the host's inflammatory response and if they fail to achieve their goal, pro-inflammatory mediators can continue, resulting in tissue injury[4]. The anti-inflammatory cytokines IL-4 and IL-10 have been implicated in NEC[4].

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF): The colony stimulating factors are a group of cytokines central to the hematopoiesis of blood cells, the modulation of their functional responses as well as the maintenance of homeostasis and overall immune competence[102]. GM-CSF is produced by a variety of cell types including macrophages, T lymphocytes, fibroblasts, endothelial cells, B lymphocytes, mast cells, eosinophils, and neutrophils[103]. In some cases, production of GM-CSF is constitutive as in a number of tumor cells lines; however, in most cases it requires stimulation of the producing cell with other cytokines, antigens, or inflammatory agents[103]. All of the biological effects of GM-CSF are mediated via the GM-CSF receptor which signals through MAPK and JAK/STAT pathways[103]. GM-CSF receptor expression is characterized by low number (20-200 per cell) and high affinity[103]. GM-CSF has pleiotropic and widespread effects on hematopoietic cells. It functions to promote the proliferation and maturation of neutrophils, eosinophils, and macrophages from bone marrow progenitors[103]. It also acts as a growth factor for erythroid and megakaryocyte progenitors in synergy with other cytokines[103]. The role of GM-CSF in cell survival results from apoptosis inhibitory mechanisms[103]. In addition to its role in the up-regulation of hematopoietic development, GM-CSF has been shown to have a profound influence on the biological functions of neutrophils, eosinophils, basophils, macrophages, lymphocytes, as well as endothelial cells[103]. These responses are widespread and point to a central role of GM-CSF in inflammation both through the direct activation of effector cells alone or in combination with other cytokines, as well as indirectly, through the stimulation of additional inflammatory mediator production[103]. Some of these biological effects include enhanced antigen presentation, chemotaxis, synthesis of a variety of soluble mediators and enzymes, release of reactive oxygen intermediates and histamines, antibody-dependent cell killing, and phagocytosis which contribute differentially to the immune defenses against bacterial, viral, fungal, and parasitic infections as well as tumor development[103]. Over-expression of GM-CSF leads to severe inflammation[104]. GM-CSF is used clinically to treat neutropenia in cancer patients undergoing chemotherapy, in AIDS patients during therapy and in patients after bone marrow transplant[103,105].

TNF-α: TNF-α release is triggered by a number of inflammatory stimuli including endotoxin (LPS), gram positive bacteria enterotoxin, viruses, fungi, and parasites[106]. Important cell sources of TNF-α in the gut are macrophages, lymphocytes, NK cells, neutrophils, endothelial cells, smooth muscle cells, intestinal epithelial cells, and enteric glia[100,106]. TNF-α exerts its effects by binding to TNF receptors[107]. Binding to the TNF receptor initiates local inflammatory responses through cell activation[107]. TNF-α is released early following injury and leads to a cytokine release cascade of IL-1β, IL-6, and IL-8[100]. It also inhibits release of glucocorticoids and the regulatory cytokines TGF-β and IL-10[106]. Some actions mediated by TNF-α include apoptosis induction, neutrophil activation, neutrophil recruitment, expression

of endothelium adhesion molecules, fever, and production and release of acute phase proteins, pro-inflammatory cytokines, NO, PGE2, matrix metalloproteases, PAF, and TXA_2[106]. The pro-inflammatory effects of TGF-α are mediated in part through NF-κB activation[106]. Elevated TNF-α has been detected in full thickness, resected bowel specimens of NEC intestine and in the plasma of babies with NEC[101]. In rat models of NEC, TNF-α induces hypotension, septic shock and severe intestinal necrosis synergistically with LPS[24]. Recently, a monoclonal anti-TNF-α antibody was demonstrated to reduce hepatic and ileal TNF-α production in a neonatal rat model of NEC[82]. Compared with other inflammatory bowel syndromes, TNF-α transcripts are lower in NEC[108]. Furthermore, studies indicate that the majority of TNF-α found in the gut lumen comes from Kupffer cells in the liver[99]. Taken together, these studies suggest that TNF-α plays a less significant role in the inflammatory cascade associated with NEC as compared with other intestinal inflammatory conditions.

IL-1β: IL-1β release is triggered by a variety of stimuli including microbial products, inflammation and TNF-α[100,109]. Important cell sources of IL-1β in the gut are macrophages, neutrophils, intestinal epithelial cells, endothelial cells, fibroblasts, dendritic cells, smooth muscle cells, and enteric glia[100,109]. IL-1β exerts its effects by binding to the IL-1 receptor and activating the transcription factor NF-κB[109]. Some actions mediated by IL-1β include macrophage activation, neutrophil recruitment, expression of endothelium adhesion molecules, fever, and production and release of acute phase proteins, IL-6, IL-8, and PGE_2[100,109]. Elevated IL-1β has been detected in full thickness specimens of NEC intestine[101]. Studies measuring plasma/serum IL-1β in NEC babies have not consistently reported elevated levels[15]. The difference in results may suggest that IL-1β is more predominant in the intestinal tissue in patients with NEC.

IL-6: IL-6 release is triggered by a variety of stimuli including microbes, microbial products, TNF-α, and IL-1β[100,110]. Important cell sources of IL-6 in the gut are macrophages, endothelial cells, and intestinal epithelial cells[110]. IL-6 exerts its effects by binding to the IL-6 receptor that signals through the STAT-4 pathway[110]. The IL-6 receptor is only expressed on hepatocytes and some leukocytes[110]. Some actions mediated by IL-6 include production of acute phase proteins, B cell growth, antibody production, T cell proliferation, and enhanced activity of hematopoietic growth factors such as GM-CSF[100,110]. Anti-inflammatory effects of IL-6 include production of tissue inhibitors of metalloproteinases and inhibition of superoxide production[111]. High levels of umbilical cord IL-6 have been associated with neonatal disease processes including NEC and systemic inflammatory response syndrome[112]. Elevated IL-6 has been reported in the plasma and stool of babies with NEC[4]. A study that looked at IL-6 mRNA expression in surgical intestine specimens from babies with NEC did not find a difference in comparison to control specimens[101]. Since IL-6 plays a dual role in inflammation it may serve as an anti-inflammatory mediator despite being correlated with increased morbidity and mortality in NEC patients.

IL-8: IL-8 synthesis and release is triggered in response to various stimuli including LPS, TNF-α, and IL-1β[113]. Important cell sources of IL-8 in the gut are macrophages, endothelial cells, intestinal epithelial cells, and fibroblasts[114]. IL-8 exerts its effects by binding to chemokine receptors CXCR1 and CXCR2 that signal through phospholipase c and PI3-kinase, respectively[113]. Some actions mediated by IL-8 are attraction of neutrophils and basophils to the site of inflammation, neutrophil activation and migration into tissues and production of acute phase proteins[114]. In intestinal specimens from patients with NEC, IL-8 mRNA expression was up-regulated, correlated with disease severity and was limited to areas with histological inflammation[4,115]. Similarly, plasma IL-8 levels are elevated in infants with NEC and levels correlate with clinical severity[4]. The vulnerability of the premature infant to develop NEC may, in part, be explained by the excessive inflammatory response shown by fetal enterocytes compared to more mature enterocytes[66]. When exposed to inflammatory stimuli, fetal intestinal cells exhibit a greater IL-8 response compared to mature intestinal cells[66]. This exaggerated response may partly be explained by the developmental down regulation of I-κB, an inhibitor of NF-κB[65].

IL-12: IL-12 synthesis and release is the early response to bacteria, bacterial products, and viruses[116]. Important cell sources of IL-12 in the gut are macrophages, neutrophils, B cells, and dendritic cells[116]. IL-12 exerts its effects by binding to IL-12 receptors present on T cells and NK cells[116]. Some actions mediated by IL-12 include IFN-γ production, Th1 and NK cell proliferation, cytotoxic T lymphocyte and Th1 cell differentiation, macrophage activation, and production of complement-fixing antibodies[116]. Several studies have identified putative NF-κB sites in the promoter regions of the IL-12 p40 genes[117]. IL-12 is a potentially important cytokine in the development of NEC. Halpern[99] localized IL-12 via immunohistochemistry to monocytes in the intestinal mucosa and lamina propria and correlated IL-12 positive cells with tissue damage in a neonatal rat model of NEC.

Interleukin-18 (IL-18): IL-18 is a cytokine that shares structural and functional properties with IL-1 and is pro-inflammatory inducing production of TNF-α and IL-1β[118]. IL-18 synthesis is triggered by LPS, Fas ligand and gram positive bacteria exotoxins[119]. Important cell sources of IL-18 in the gut are macrophages, dendritic cells, and intestinal epithelial cells[119]. IL-18 exerts its effect by binding to the IL-18 receptor present on macrophages, neutrophils, NK cells, endothelial cells, smooth muscle cells, and lymphocytes[119]. IL-12 upregulates the IL-18 receptor on lymphocytes[119]. Binding to the IL-18 receptor results in NF-κB activation. Some actions mediated by IL-18 include IFN-γ production,

enhanced NK cell cytotoxic activity, B cell antibody production, macrophage production of IL-8, activation and migration of neutrophils, phagocytosis, and integrin expression[119]. IL-18 can promote Th1 or Th2 lineage maturation depending on the underlying genetic influence and cytokine environment. The risk of NEC has been associated with the frequency of the IL-18^{607} AA genotype[19]. Recent data imply that IL-18, in the absence of IL-12, may facilitate the development of Th2 responses[118]. IL-18 is also essential to host defense against a variety of infections[119] and is potentially important in the development of NEC. Immunohistochemistry reveals the presence of IL-18 in intestinal epithelial and lamina propria cells which correlates with the degree of tissue damage in a neonatal rat NEC model[99]. Depending on the surrounding environment, IL-18 may play a destructive or protective role in NEC.

IL-4: IL-4 is a pleiotropic, immunoregulatory cytokine produced by Th2 cells, mast cells, B cells, and stroma cells[100,120]. IL-4 displays a wide variety of effects on B cell growth and differentiation, T cell growth and regulation, hematopoietic cells and differentiation of $CD4^+$ T cells into Th2 cells and is a key regulator in humoral and adaptive immunity[100,120]. IL-4 induces B cell class switching to IgE and upregulates MHC class Ⅱ production[100,120]. IL-4 is known to promote Th2 type responses and to exert immunosuppressive effects on macrophages including the suppression of pro-inflammatory cytokine production[100,120]. Although data are not available about the importance of IL-4 in NEC, isolated lamina propria mononuclear cells from the inflamed intestinal mucosa of children with chronic inflammatory bowel disease express and secrete IL-4 in lower amounts than control cells[121]. In preliminary studies, VLBWI with NEC were shown to be less likely to possess the IL-4 receptor α-chain mutant allele compared to infants without NEC[19]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced Th2 state[19]. It is speculated that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC[19].

IL-10: IL-10 is the most important regulatory cytokine in the intestine and is primarily synthesized by Th2 cells, monocytes, and B cells[120]. Mononuclear production of anti-inflammatory mediators such as IL-10 is diminished in the newborn when compared to the adult, with preterm infants synthesizing less than term infants[122,123]. It is postulated that this phenomenon allows for persistent up-regulation of the inflammatory response and therefore increased susceptibility in the preterm neonate to long-term tissue damage after acute inflammatory conditions[124]. Interleukin-10 has been implicated as an inhibitor of pro-inflammatory cytokine production and of several accessory cell functions of the macrophage, T cell and natural killer (NK) cell lines[120]. Kuhn[125] demonstrated that IL-10 deficient knockout mice were predisposed to developing inflammatory colitis, suggesting that IL-10 works to counterbalance the response to enteric inflammatory stimuli. In fact, intraperitoneal IL-10 injections in a mouse model of ischemia/reperfusion injury reduced local and systemic inflammatory reactions[126]. Edelson[15] noticed significantly increased concentrations of IL-10 with severe NEC. IL-10 has also been shown to decrease the production of metalloproteinases[127] and suppress iNOS mRNA and NO expression in small bowel, liver and serum[128]. These findings indicate that IL-10 is a strong counter regulatory cytokine and that the potential of IL-10 to provide therapy in the setting of NEC is high. Perhaps the high levels of IL-10 in severe NEC are the body's response to dampen the inflammatory response.

ROS

One of the major endogenous sources of ROS in the intestine is the xanthine dehydrogenase/xanthine oxidase (XD/XO) system[129]. Xanthine dehydrogenase (XD), the precursor of XO, is constitutively and abundantly expressed in the intestinal villus epithelium, which catalyzes the conversion of hypoxanthine to xanthine, coupled with the reduction of NAD^+ to NADPH[130]. Because XO uses molecular oxygen rather than NAD^+ as an electron acceptor and thereby generates superoxide, XD to XO conversion (during ischemia) has been suggested to play the central role in intestinal reperfusion injury[129]. Following PAF challenge, it is the ileum that shows the most dramatic XD to XO conversion[130]. The central role of XO and ROS in causing the injury is supported by pre-treatment with allopurinol, a xanthine oxidase inhibitor, which largely prevents PAF-induced bowel necrosis[131]. Infusion of superoxide dismutase plus catalase also alleviates the injury[131]. In a piglet model of NEC, the level of the tissue antioxidant, α-tocopherol (vitamin E) was low in formula compared to colostrum fed piglets[13]. Thus, infants with NEC are under oxidative stress and may benefit from exogenous sources of antioxidants to replenish limited supplies.

NEC models

There are a number of accepted models used to study NEC and the cytokine cascade. These models serve to create necrotic bowel in animals to simulate that in the newborn child. LPS, PAF and TNF-α are often used to create intestinal ischemia. LPS is thought to mimic the bacterial overgrowth in the intestinal lumen and PAF and TNF-α cause a hypotensive response and shock[24].

Many animal models can simulate NEC, but often do not contain the aspect of prematurity that is seen in human NEC. The most physiological animal model that most closely resembles human NEC entails removing rat pups from the maternal uterus, exposing them to maternal milk, and stressing them with asphyxia, gram negative bacteria colonization, and artificial formula feedings[132]. After a few days of life, the rat pups begin to exhibit signs of NEC including intestinal distension and bloody diarrhea.

Other models have been described that do not physiologically resemble human NEC, but aid in the study of the disease process. These include inducing hypoxia for 5 min followed by 10 min with 100% oxygen[133], hypoxia for 50 s followed by cold exposure[134], superior

mysenteric artery clamping with or without PAF[135], intraarterial injection of TNF-α[136], and placing rats into a 100% nitrogen or 10% oxygen environment[24]. Finally, a rat model has been described by Chan[137] who created intestinal ischemia by increasing intraluminal pressure and injecting *E. coli* into the lumen.

In addition to *in vivo* animal models, various *in vitro* models have been created. The cell lines are often intestinal-derived and immortal such as CaCo-2, a human colon carcinoma cell line[138]. Inflammatory stimulants such as LPS and pro-inflammatory cytokines can be added to cell cultures which can then be analyzed to determine the presence or absence of specific cytokines. In addition, cells can be studied with regards to permeability, viability and expression of inflammatory markers after addition of certain stimulants or creation of hypoxic environments. Paracellular conductance can be assessed by measuring both trans-epithelial resistance (TER) and determining the rate of permeation of radiolabelled, hydrophilic probes between mucosa and serosa compartments of vertical diffusion chambers. It is unfortunate that primary cultures of human enterocytes have a limited life span (hours) in culture and therefore have not been useful as a model.

Symptomatic treatment and surgery

Due to the limited understanding of the fundamental biological processes that underlie the development of NEC, there is no cure for this devastating pediatric disease[21]. Symptomatic treatment of the infant with NEC begins with prompt recognition of the diagnosis and medical stabilization[2,9]. The treatment of NEC is based on the severity of the disease and is directed toward reduction of factors that aggravate the disease, treatment of infection and support of respiratory and cardiovascular systems[139]. Blood pressure should be closely monitored, all enteral feedings and medications should be discontinued and decompression of the gastrointestinal tract with placement of a gastric tube should proceed to evacuate residual air and fluid[9]. Rapid volume expansion with isotonic fluids may be necessary to reverse hypotension as well as frequent monitoring of blood glucose levels[9]. An intravenous infusion of total parenteral nutrition should begin during the 10-14 d bowel rest period[9]. The reinstitution of feedings generally is done in a slow and cautious manner, using an elemental formulation to allow for optimal absorption of all nutrients and to avoid further potential injury to the intestinal mucosa[7]. Broad-spectrum antibiotics including ampicillin and an aminoglycoside should be started as soon as cultures have been obtained[139]. With the increasing prevalence of infections from coagulase-negative staphylococcus, vancomycin may be used instead of ampicillin[2]. Anti-microbial choices should be guided by local resistance patterns[2,139]. Adjunctive therapy includes cardiovascular support (volume expansion with packed red blood cells), pulmonary support (oxygen and ventilation), and hematological support (blood product transfusion) as clinically indicated[1,139]. Indications for surgical intervention include peritoneal free air and signs of intestinal perforation[9]. Surgical intervention frequently results in resection of areas of necrotic bowel and exteriorization of viable ends (multiple ostomies) to allow for continued bowel decompression[2,9]. Recently, primary peritoneal drainage has been proposed as an alternative to surgical treatment. NEC STEPS and NET, prospective multi-centre randomized controlled trials, are currently underway to examine the effectiveness of primary peritoneal drainage versus laparotomy as primary therapy for perforated NEC in VLBW infants[140,141].

Table 4 Strategies to prevent necrotizing enterocolitis

Evidence-based support for efficacy	Limited data to support efficacy
Breast feeding	Cautious advancement of feedings
Trophic feeding	Fluid restriction
Antenatal steroids	Oral immunoglobulins
Enteral administration of antibiotics	L-arginine supplementation
	Polyunsaturated fatty acids
	Acidification of milk feeds
	Probiotics, prebiotics and postbiotics
	Growth factors and erythropoietin
	Free radical scavengers

PREVENTION

Strategies to prevent NEC fall into two major categories: Those with probable or proven efficacy and those that are experimental with unproven efficacy[2] (Table 4).

The most effective preventative strategies should improve both short-term and long-term outcomes for VLBW preterm infants and address the problems of prematurity.

Human milk

Human milk has been reported to reduce the incidence of NEC by up to 10 fold compared with infant formula whether using mother's own or donor milk[142]. Breast milk also reduces the severity of NEC[8]. The protective effect of breast milk has been correlated with its anti-inflammatory components (IL-10), growth factors (EGF), erythropoietin (Epo), lysozyme, immunoglobulins as well as pre- and probiotics that modulate intestinal microflora composition to the advantage of the host[55,143,144]. Research looking at a gut-stimulation, or gut-priming protocol has demonstrated potential benefits of promoting maturation of the gut by introducing early feedings with human milk[3]. The activity of acetyl hydrolase (PAF-AH), an enzyme that degrades PAF, is lower in neonates under 3 wk of age than at any other time[145,146]. The additional presence of PAF-AH activity may also partly explain the protective effect of breast milk, as infant formulas do not contain it[8]. Whether preterm human milk reduces the incidence of NEC is not clear at present[8]. Despite its advantages, it is important to appreciate that human milk alone will not eliminate NEC as cases are reported in neonates who have been breast-fed exclusively with human breast milk[8].

Trophic feeds

Initiation of trophic feeds, small volumes of breast milk

or formula, may overcome gut atrophy and inflammatory responses associated with prolonged bowel rest. Trophic feeds improve the activity of digestive enzymes, enhance the release of digestive hormones and increase intestinal blood flow and digestive motility in premature infants[147]. In addition, infants given early trophic feeds seem to have better feeding tolerance, improved growth, reduced period of hospitalization and decreased likelihood of sepsis compared with infants who are not[147]. Furthermore, early trophic feeds do not increase susceptibility to developing NEC. However, studies have not yet clearly delineated the best feeding strategies for premature infants[147].

Antenatal glucocorticoids

Antenatal glucocorticoid therapy has beneficial effects by suppressing inflammation and promoting gastrointestinal maturation and function including reduced mucosal uptake of macromolecules, decreased colonization with aerobic bacteria, reduced bacterial translocation, and increased activity of enzymes such as lactase, maltase, sucrase, and Na/K-ATPase[148,149]. A significant reduction in the incidence and risk of NEC following antenatal glucocorticoid therapy has been reported in several large, randomized control trials[150,151]. Mortality rate was also lower and there were fewer indications for surgical intervention[152]. Antenatal glucocorticoids have been reported to alter immune system development in very premature infants[153]. Mothers with the presence of infection or a condition that may compromise blood flow to the fetus (ex. pre-eclampsia) during pregnancy may be at risk of delivering a premature baby and may potentially benefit from early use of glucocorticoids. Thus, antenatal glucocorticoid therapy is a simple and effective strategy for global prevention of NEC and more research should be done to investigate potential impact on development.

Enteral antibiotics

Enteral antibiotics have been used as prophylaxis against NEC in low birth weight and preterm infants given the role of bacterial colonization in the pathogenesis of the illness. A systemic review and meta-analysis has reported that the administration of prophylactic enteral antibiotics resulted in significant reduction in NEC[154]. The trend towards a reduction in deaths was not significant[154]. The possible harmful effects of prophylactic antibiotics including the development of bacterial resistance and alteration of the natural microflora make it difficult to recommend this strategy for prevention of NEC.

Standardized feeding regimens (cautious advancement of feedings)

Inter-centre differences in clinical practice involving feeding regimens are significant factors linked to the prevalence of NEC in VLBW neonates[155]. A relationship between the rate of feeding advancement and an increased incidence of NEC exists[156]. A significant decline of 87% in the incidence of NEC and 29% in the risk of developing NEC was reported following implementation of a standardized feeding regimen in the form of clinical practice guidelines[157,158]. Parenteral nutrition coupled with minimum enteral feeding is the approach commonly advocated for the initial nutritional management of high risk infants and helps protect against NEC[159].

Fluid restriction

Excess fluid has been implicated in the pathogenesis of NEC. A systemic review and meta-analysis indicates that restricted water intake significantly increases postnatal weight loss and significantly reduces the risk of NEC[160]. Careful restriction of water intake (meeting the physiological needs without allowing significant dehydration) could be expected to decrease the risk of death from NEC without significantly increasing the risk of adverse consequences.

Probiotics

Since bacterial colonization can affect the course of many intestinal diseases, probiotics are emerging as a promising therapy. Probiotics are living microorganisms, which upon administration in sufficient numbers colonize the gut and exert health benefits beyond basic nutrition on the host[161]. As components of infant formula, typically used probiotic microorganisms are members of the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces* and to a lesser extent *Streptococcus*. The beneficial effects of probiotics range from changes in intestinal permeability and enhanced mucosal IgA responses to an increased production of anti-inflammatory cytokines and protection of the mucosa against colonization from pathogens[162]. *Bifidobacteria* are the most common organisms recovered from the gastrointestinal tract of breast-fed neonates. Given the role of inappropriate gastrointestinal colonization by bacteria in the pathogenesis of NEC, probiotics may be beneficial in the prevention of NEC. Several studies have used different strains of probiotics and different administration regimens (length of treatment and dose) in preterm infants. None of the trials have reported adverse effects and no episodes of pathogenic infection caused by a probiotic organism have been observed[78,79,163,164]. Clinical trials show that probiotic supplements (*Lactobacillus acidophilus*, *Bifidobacterium infantis, Bifidobacterium bifidus,* and *Streptococcus thermophilus*) reduce the incidence and severity of NEC[78,79,165]. Larger clinical trials are necessary to confirm the safety and efficacy of this promising intervention to better define the benefits and risks for premature infants before wider use can be recommended.

Prebiotics

Another potential preventative strategy is to administer prebiotics, non-digestible dietary supplements, such as long chain carbohydrates or mucins, which promote proliferation of beneficial commensal bacteria[166]. Preliminary studies show increased *Bifidobacterium* stool colonization and decreased pathogenic bacterial colonization in preterm infants fed with formula containing prebiotics (90% short chain galacto-oligosaccharide, 10% long chain fructo-oligosaccharide) compared with infants fed control formula[167]. Furthermore, prebiotic treatment may have a positive effect on host immune function[168]. Because prebiotic supplements do not contain live microorganisms, they carry less risk of infection than

probiotic therapies. However, prebiotic administration has been associated with unwanted (but reversible) side effects such as flatulence, bloating and diarrhea[166].

Postbiotics

Another potential therapy involves bacterial metabolites or postbiotics, such as butyric acid, a short-chain fatty acid produced by commensal bacteria in the colon through anaerobic catabolism of complex carbohydrates. Butyrate is a major energy source for colonic enterocytes and has a widely recognized but poorly understood role in intestinal growth and differentiation[169,170], inflammatory suppression[171] and apoptosis[172]. Butyrate and other small molecule products might generate some of the beneficial effects of the normal flora (and exogenous probiotics and prebiotics), and could be a safe alternative therapeutic strategy. Butyrate has been administered with limited success in human inflammatory bowel disease[173], but there are as yet no studies in neonates.

Other products of commensal bacteria can induce protective responses that promote intestinal health. The beneficial effects of probiotic bacteria can be replicated by treatment with isolated microbe-associated molecular patterns (MAMPs)[174]. A MAMP is a molecular sequence or structure in any pathogen-derived molecule that is perceived via direct interaction with a host defense receptor[175]. For example, in mice unmethylated probiotic DNA ameliorates colitis[174]. Oral administration of inactivated probiotics (heat-killed commensals) or bioavailable toll-like receptor ligands could potentially induce beneficial TLR-mediated protective effects without carrying the infectious risk of probiotic therapies.

Arginine supplementation

Endothelial nitric oxide is an anti-inflammatory agent and vasodilator that is involved in the maintenance of intestinal vascular permeability, mucosal integrity and barrier function[21,86]. The plasma levels of the amino acid arginine, a substrate for NOS, have been shown to be low in neonates with NEC[176,177]. Arginine supplementation has recently been shown to reduce the incidence of all stages of NEC in a randomized, double blind, placebo controlled trial in preterm neonates[178]. Whether the beneficial effects of arginine supplementation in prevention of NEC are related to synthesis of glutamine or to its free radical scavenging action is currently unknown[179,180]. Guidelines have not been established for the safety and efficacy of L-arginine at doses above standard dietary practices in NEC[181].

Free radical scavengers (anti-oxidants)

Free radicals have been implicated in several neonatal disease processes including NEC[182]. A human recombinant superoxide dismutase is currently available and has been shown to prevent damage and attenuate eicosanoid release in a rabbit model of NEC[183,184]. The anti-oxidant vitamin E has been shown to reduce lipid peroxidation and intestinal lesions in a neonatal rat model of NEC induced by hypoxia-ischemia[183,184]. More studies on the therapeutic role of anti-oxidants in NEC should be done.

Acidification of gastric contents

Preterm neonates are often hypochlorhydria and enteric, Gram negative bacteria often colonize their stomachs, especially after gavage feeding[185]. Carrion and Egan[186] have documented that acidifying the feedings of preterm neonates to a pH low enough to inhibit gastric bacterial proliferation significantly lowers the risk and incidence of NEC.

Polyunsaturated fatty acids

Phosphotidylcholine (PC) is a major phospholipid constituent of mucosal membranes and the fatty acid component of PC, arachidonic acid, is a substrate for intestinal vasodilatory and cytoprotective eicosanoids[8]. Long chain polyunsaturated fatty acids (PUFA) have been proposed to modulate inflammation and immunity[187]. A clinical trial of formula feeds with or without supplementation with PUFA in the form of egg phospholipids in preterm neonates showed that the supplemented formula contained 7-fold more arachidonic acid and docosahexanoic acid and reduced the incidence of stage Ⅱ and Ⅲ NEC[188]. Recent evidence from an experimental study indicates that the protective effect of long chain PUFA is mediated by modulation of PAF metabolism and endotoxin translocation[189].

Oral immunoglobulins

A number of reports have been published, which suggest that oral immunoglobulins (IgA and IgG) have an immunoprotective effect on the gastrointestinal mucosa[190,191]. Premature infants have decreased levels of immunoglobulins, especially secretory IgA[192]. A reduction in the incidence of NEC following feeding an oral IgA-IgG preparation was reported as early as 1988[190]. A systemic review on oral immunoglobulin for the prevention of NEC did not show a significant reduction on the incidence of definite NEC[193]. No randomized controlled trials of oral immunoglobulins for the prevention of NEC have been carried out. Current evidence does not support the administration of oral immunoglobulin for the prevention of NEC.

EGF

EGF is a growth factor that exerts its effects by binding to the EGF receptor. EGF is an important constituent of gastrointestinal secretions and has multiple effects upon gut epithelial cells including cytoprotection, stimulatory effects on cell proliferation and migration, induction of mucosal enzyme and trefoil peptide expression, and inhibitory effects on gastric acid secretion[48]. Preterm neonates with NEC have diminished levels of salivary and serum EGF[194]. The presence of immunoreactive EGF receptors in gut epithelial cells of preterm neonates with NEC raises the possibility of using EGF for prophylaxis or treatment of NEC[195]. In a neonatal rat model of NEC, EGF treatment maintained intestinal integrity at the site of injury by accelerating goblet cell maturation and mucin production and normalizing expression of tight junction proteins[196]. Researchers have already warranted that the clinical use of EGF may be associated with a variety of problems and side

effects and that careful selection of patients and evaluation of risk-benefit ratios are necessary[197]. Given the potential for adverse effects and the fact that maturity alone is not a protective factor for NEC the use of any growth factors in preterm neonates warrants extreme caution.

Epo

The presence of Epo in human milk and the expression of Epo receptors on intestinal villous enterocytes of neonates suggest that Epo has a role in growth and development of the gastrointestinal tract[198-200]. Ledbetter[200] administered recombinant Epo for the prevention and treatment of the anemia of prematurity and demonstrated that the rEpo group had a lower incidence of NEC. Akisu[133,201] indicated that rEpo decreased lipid peroxidation but not PAF generation. Although not completely absorbed, Epo acts as a trophic factor in developing rat bowel whether given enterally or parenterally[199]. Current evidence indicates that the protective effect of rEpo may be related to inhibition of NO formation[202].

CONCLUSION

A variety of other experimental agents have been studied in search for an effective agent for the prevention of NEC. These include anti-TNF-α[82], PAF receptor antagonists[203], heparin-binding EGF-like growth factor[204], anti-inflammatory cytokines (IL-10)[205], pentoxifylline[206], intestinal trefoil factor 3[207], and glucagon-like peptide 2. Recent research has identified that complex glycosphingolipids in the form of gangliosides act as bioactive factors down-regulating many acute pro-inflammatory signals in the intestinal mucosa. Perhaps the solution to NEC will involve identification of an intestinal control mechanism that optimizes (or disregulates) the balance between pathways that signal inflammation, hypoxia, and mucosal cell growth or metabolism.

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GASTRIC CANCER

Effect of *NHE1* antisense gene transfection on the biological behavior of *SGC-7901* human gastric carcinoma cells

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Abstract

AIM: To study the effect of type 1 Na^+/H^+ exchanger (*NHE1*) antisense human gene transfection on the biological behavior of gastric carcinoma cell line SGC-7901.

METHODS: Antisense *NHE1* eukaryotic expression on vector pcDNA3.1 was constructed by recombinant DNA technique and transfected into gastric carcinoma cell line *SGC-7901* with DOTAP liposome transfection method. Morphological changes of cells were observed with optic and electron microscopes. Changes in cell proliferative capacity, apoptosis, intracellular pH (pH_i), cell cycle, clone formation in two-layer soft agar, and tumorigenicity in nude mice were examined.

RESULTS: Antisense eukaryotic expressing vectors were successfully constructed and transfected into *SGC-7901*. The transfectant obtained named *7901*-antisense (*7901-AS*) stablely produced antisense *NHE1*. There was a significant difference between the pH_i of *7901-AS* cells (6.77 ± 0.05) and that of *7901-zeo* cells and SGC-7901 cells (7.24 ± 0.03 and 7.26 ± 0.03, $P < 0.01$). Compared with *SGC-7901* and *7901-zeo* cells, *7901-AS* cells mostly showed cell proliferation inhibition, G_1/G_0 phase arrest, increased cell apoptotic rate, recovery of contact inhibition, and density contact. The tumorigenicity in nude mice and cloning efficiency in the two-layer soft agar were clearly inhibited.

CONCLUSION: *NHE1* antisense gene significantly restrains the malignant behavior of human gastric carcinoma cells, suppresses cell growth and induces cell apoptosis, and partially reverses the malignant phenotypes of *SGC-7901*. These results suggest a potential role for human tumor gene therapy.

Key words: *NHE1* gene; Eukaryotic expression vector; Antisense gene therapy; Gastric cancer

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INTRODUCTION

The type 1 Na^+/H^+ exchanger (NHE1) is a transmembrane protein found in all eukaryotic cells. One of its functions is to remove excess H^+ in the cytoplasm by Na^+-H^+ exchange system, resulting in a stable intracellular pH level[1,2]. The process of glycolysis induces the tumor cells to produce large quantities of lactic acid and H^+. There is vigorous Na^+-H^+ exchange in the tumor cells, dependent upon the enhanced expression of NHE1 membrane protein. Most of the H^+ pumped out of the cells prevents the intracellular acidification of tumor cells, resulting in the protection of tumor cells from apoptosis[3,4]. Previous studies have shown that NHE1 protein expression in gastric carcinoma tissues was significantly higher compared to that in normal gastric mucosa and precancerous lesions[5]. Therefore, inhibition of up-regulation of *NHE-1* gene expression in human gastric carcinoma cells may induce intracellular acidification, resulting in apoptosis of tumor cells, which is helpful in the treatment of such tumors. In the present study, we constructed the antisense *NHE-1* eukaryotic expression vector and transfected it into gastric carcinoma cell line *SGC-7901* in order to investigate the effects of antisense *NHE1* on malignant biological behavior of the gastric carcinoma cell line *SGC-7901*.

MATERIALS AND METHODS

Cell line and cell culture

The human gastric carcinoma cell line *SGC-7901* was

used in this study. The cells were grown in RPMI1640 medium (Sigma, St Louis, MO, USA), supplemented with 10% FBS (fetal bovine serum) and antibiotics(100 μg/mL streptomycin and 100 U/mL penicillin) in an atmosphere consisting of 5% CO_2 in air at 37℃ in an humidified incubator.

Gene transfection

pEAP cloning vector of human *NHE1* cDNA was kindly provided by Dr. Josset Noel (Montreal University, Canada). Eukaryotic expression vector *pcDNA3.1* (-)/*Zeo* and Zeocin were obtained from Invitrogen Co. The experimental procedures of gene transfection were carried out according to the directions of DOTAP™ liposome transfection kit (Roche Diagnostics, Mannheim, Germany). The cells were plated at a density of 1.5-3 × 10^5 cells/35 mm dish and were grown for 24 h. The cells were transfected for 6 h with 2.5 μg plasmid DNA and 15 μL DOTAP in 2 mL of RPMI1640 medium without FBS and antibiotics. The cells were recovered for 48 h in the medium with 10% FBS. The stable transfectant was maintained in 100 μg/mL Zeocin (Invitrogen) in the medium for at least 20 d. The antisense *NHE1* and the control plasmid transfectant were named *7901-AS* and *7901-zeo*, respectively.

Analysis of exogenous genes integration

In order to identify exogenous integration in the nucleic DNA of *SGC-7901* cells, the Zeomycin-resistant antibiotic-selecting gene (*Zeo*R) was amplified by polymerase chain reaction (PCR) assays with a ZeoR-specific primer set (5'-GGCCAAGTTGACCAGTGC-3' as forward and 5'-GTCAGTCCTGCTCCTCGG-3' as backward). DNA was extracted from *SGC-7901* and the transfected cells using standard techniques. The total volume of the PCR reaction system was 25 μL, containing 0.2 mmol/L dNTP, 1 mmol/L of each primer, 2U *Taq* polymerase, 1 × reaction buffer and 100 ng DNA template. After predegeneration at 94℃ for 5 min in a PE cycle, 30 PCR cycles were performed at 94℃ for 30 s, 53℃ for 30 s, and 72℃ for 30 s, then extended at 72℃ for 5 min in a PCR thermocycler (Pekin-Elmer Cetus, Norwolk, CA, USA). Electrophoresis was performed on 1% agarose gel, and the findings were observed and pictures taken under the Burdick lamp.

Determination of intracellular pH

High-concentration potassium-buffer containing the following chemicals (in mmol/L): 90-130 KCl, 5 NaCl, 1 $MgSO_4$, 1 $CaCl_2$, and 10 HEPES was infused into 6 tubes (5 mL in each), with pH values adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. Nigericin (30 μmol/L) and BCECF (0.25 μmol/L) were added to the 6 tubes. At the logarithm growth phase, the cells were digested with pancreatin to prepare a single-cell suspension. The cells were washed twice with PBS, and an identical number of cells were added to the 6 tubes for inoculation at 37℃ for 12 min. The cells were stimulated with argon ion laser at 488 nm. The emitted fluorescence was recorded at 530 nm and 640 nm and the ratio (FIR) was calculated. The ratio curve and the standard curve of pH were drawn.

Cells at the logarithm growth phase were digested with pancreatin, and centrifugated for 5 min at 1000 r/min. After removal of the supernatant, the cells were washed once using saline. BCECF/AM stock solution (2 mg/mL), prepared with anhydrous DMSO, was added to the serum-free and phenolsulfonphthalein-free medium until the BCECF concentration reached 0.25 μmol/L. Cells in the stock solution were incubated for 12 min for 1 h at room temperature. The cells were stimulated with argon ion laser at 488 nm and the emitted fluorescence at 530 and 640 was recorded, and the ratio (FIR) was calculated. Intracellular pH (pH_i) was calculated according to the standard curve.

Cell morphology and growth features observation

The shape, size and growth features (such as contact inhibition, density inhibition and anchorage-dependence) of *7901-AS* cells were observed using invert microscopy and common microscopy after hematoxylin and eosin staining. Cell proliferation speed was assayed by the 3-(4,5-dimethyl-2 thiazoyl)-2,5-dipheny-2H-tetrazolium bromide method.

Cell cycle and apoptosis rate analysis

Exponentially growing cells were collected and fixed with 75% cold ethanol for at least 24 h and were analyzed for cell cycle distribution and apoptosis rate by DNA content analysis using propidium staining under flow cytometry (FACS420).

Double-deck soft agar colony forming efficiency

The cells were planted in 24-hole plastic plates spread with low melting point agar (0.35% in upper layer and 0.6% in low layer). Each type of cell was planted in five holes, with 1000 cells in each hole. The cells were cultivated at 37℃, with 5% CO_2 and under saturation humidity for 2-3 wk. Cells that were larger than 75 μm in diameter or clones with more than 50 cells were counted under an invert microscope. Clone form-rate was calculated according to the clone form number/inoculation cell number × 100%.

Tumorigenicity assay in nude mice

Nine Balb/c athymic 4-5 wk old female nude mice, bred in specific pathogen free conditions (purchased from the Experimental Animal Center of the Third Military Medical University, Chongqing, China) were randomly divided into three groups. Three different cell lines (*SGC-7901, 7901-zeo*, *7901-AS*), suspended in 0.1 mL serum-free RMPI 1640, were injected subcutaneously in a dose of 4-8 × 10^6 cells at two different sites of the mice. The tumor diameter was measured every 7 d. The animals were monitored regularly for tumor occurrence and size for at least 2 mo.

Statistical analysis

The findings are depicted as mean ± SD. Variance analysis and *t*-test for non-matched data were performed using a professional SPSS statistical program.

RESULTS

Construction and identification of NHE1 antisense eukaryotic expression vector

Human *NHE1* cDNA (3.6 Kb) digested from plasmid

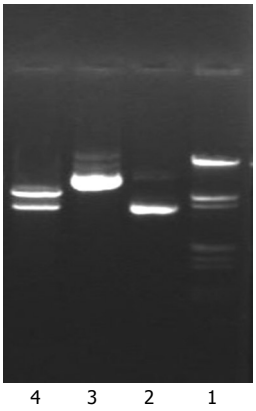

Figure 1 Identification of antisense human *NHE1* cDNA vector digested by restriction endonuclease. Lane 1: Marker (λDNA/*Eco*R Ⅰ + *Hind*Ⅲ); Lane 2: *pcDNA*3.1 (-)/*Zeo* plasmid; Lane 3: *pcDNA*3.1-*NHE*1 plasmid; Lane 4: *pcDNA*3.1-*NHE*1 plasmid digested by *Eco*R Ⅰ and *Hind*Ⅲ (5.0 and 3.6 kb).

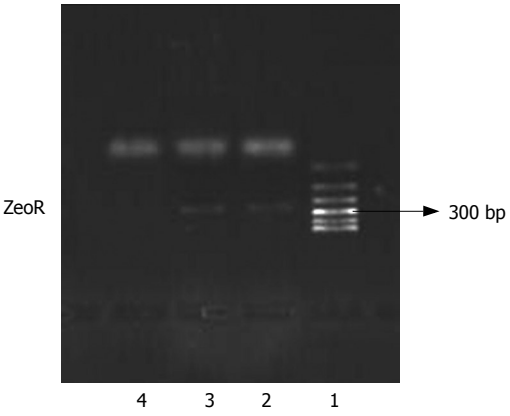

Figure 2 Analysis of exogenous gene integration in nucleic DNA of *SGC-7901*. Lane 1: Polymerase chain reaction marker; Lane 2: *7901-zeo*; Lane 3: *7901-AS*; Lane 4: *SGC-7901*.

pEAP with *Eco*R Ⅰ and *Hind*Ⅲ restriction endonuclease was inserted into the eukaryotic expression vector in antisense orientation. The recombinant DNA was further shown to be the same as designed by restriction analysis (Figure 1). A fragment of 3.6 Kb was released after digestion of the recombinant plasmid with *Eco*R Ⅰ and *Hind*Ⅲ, suggesting that the target fragment was successfully inserted into the expression vector.

The antisense *NHE1* eukaryotic expression vector was named *pcDNA3.1-NHE1*.

Identification of transfection

We introduced an antisense *NHE1* cDNA sequence into the *SGC-7901* cell line. Following Zeocin selection, drug-resistant individual clones were randomly collected from cultures infected with *pcDNA3.1-NHE1* (*7901-AS*). For controls, drug-resistant clones were selected from the cultures infected with an empty vector *pcDNA3.1* (-)/*Zeo* (*7901-zeo*). The expression of zeocin resistant gene (*ZeoR*) could be amplified in *7901-AS* or *7901-zeo* cells, but not in untransfected cells. This result suggested that exogenous genes had been integrated into the nucleic DNA of SGC-7901 cells (Figure 2).

Determination of intracellular pH

The standard curve drawn according to the buffer is shown in Figure 3. The regression equation was $y = 0.2144\chi - 0.4248$ ($r = 0.96$).

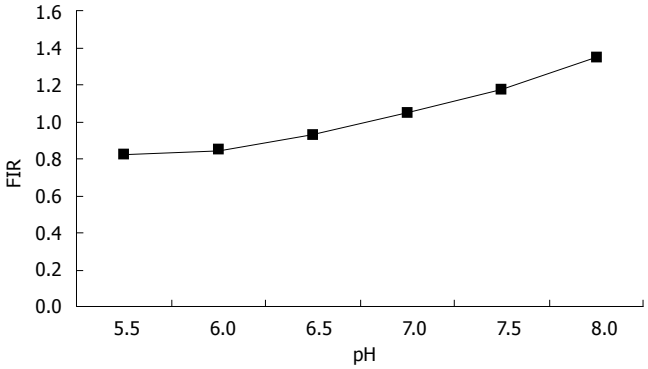

Figure 3 Standard curve of intracellular pH.

Table 1 Changes in the intracellular pH values of *SGC-7901* gastric carcinoma cells before and after transfection

Cells	Intracellular pH values determined each time			Intracellular pH (mean ± SD)
	1	2	3	
7901-AS	6.73	6.75	6.83	6.77 ± 0.05[b]
7901-zeo	7.20	7.26	7.25	7.24 ± 0.03
SGC-7901	7.22	7.28	7.28	7.26 ± 0.03

[b]$P < 0.01$ *vs* *7901-zeo* and *SGC-7901*.

The cells were stimulated with argon ion laser at 488 nm and the emitted fluorescence at 530 nm and 640 nm was recorded and the ratio of 530/640 was calculated. The intracellular pH (pH_i) was calculated according to the standard curve. The pH_i of *7901-AS* cells was 6.77 ± 0.05, whereas the pH_i of *7901-zeo* and *SGC-7901* cells were 7.24 ± 0.03 and 7.26 ± 0.03, respectively. There was a significant difference between the pH_i of *7901-AS* cells and that of *7901-zeo* cells and *SGC-7901* cells ($P < 0.01$). The reduced pH_i of *7901-AS* cells suggested that the transfected antisense *NHE1* gene successfully inhibited the expression of *NHE1* gene and blocked excessive exchange of Na^+-H^+, resulting in intracellular acidification. There was no significant difference in the pH_i of *SGC-7901* and *7901-zeo* cells (Table 1).

Morphology and growth features of 7901-AS cells

Compared with their parental cells and *7901-zeo* cells, antisense *NHE1*-transfected *7901* cells displayed several morphological changes under light microscopy, such as decreased mitotic figures, multinucleate giant cells, giant nucleate cell numbers, and nucleus:cytoplasma ratio. The *7901-AS* cells only grew in a monolayer and at low cell density. The cell proliferation slowed down on the sixth day when *7901-AS* cells grew into complete confluence (Figures 4 and 5). Further study showed that the parent cells and *7901-zeo* cells grew in clusters with large numbers of big clones. The formation rate was 11% for *SGC-7901* cells and 9.5% for *7901-zeo* cells after 2 wk culture in soft agar. By contrast, the *7901-AS* cells were scattered in soft agar with less number of clones and the formation rate was only 2%.

Cell-cycle distribution and apoptotic rate of 7901-AS cells

The *7901-AS* cells showed increased apoptotic cells and

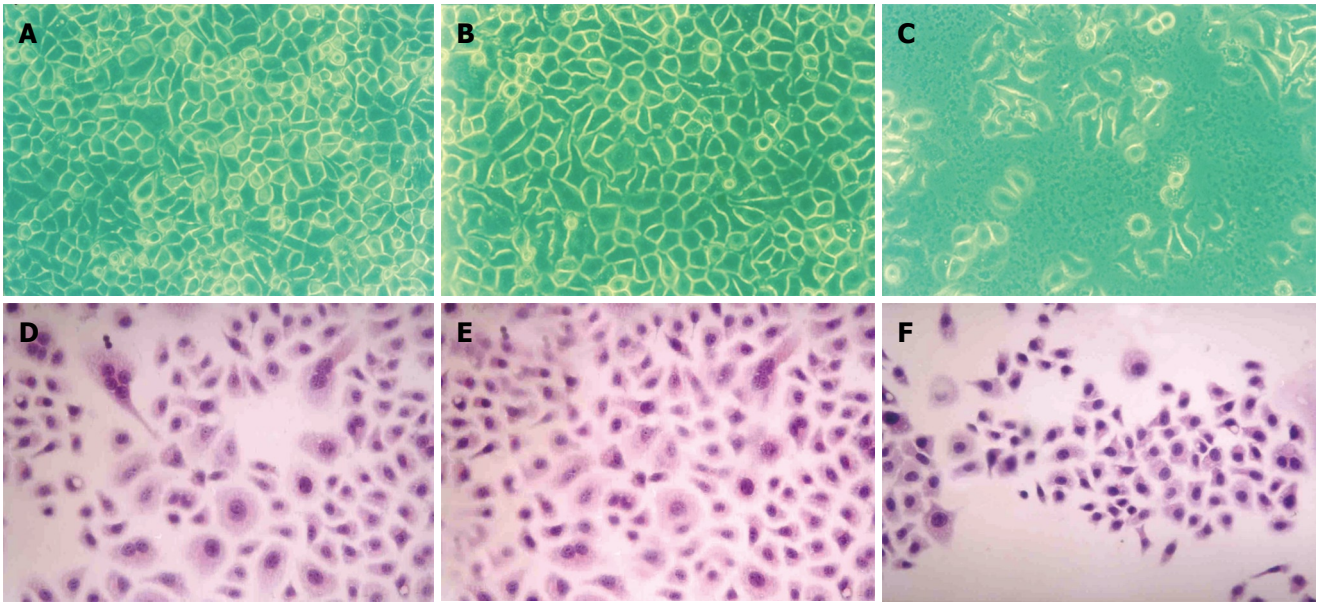

Figure 4 Morphorlogical changes in the transfected cells and parental cells. The first row was observed under inverted microscope. The second row was stained with hematoxylin and eosin, and examined by light microscopy. (**A**) and (**D**) *SGC-7901*; (**B**) and (**E**) *7901-zeo*; (**C**) and (**F**) *7901-AS*.

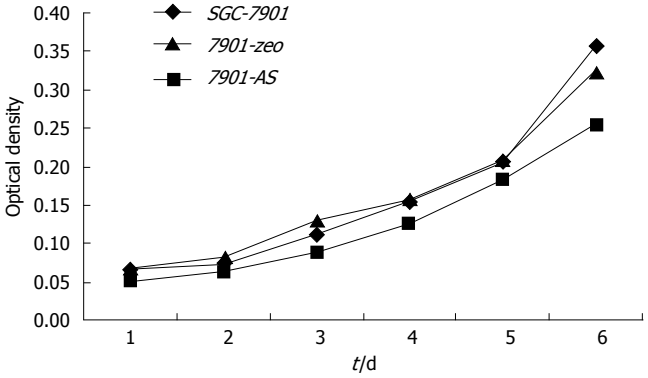

Figure 5 Comparison of growth curves of transfected cells and parental cells.

G_0/G_1 cells, and decreased S and G_2M cells, and reduced proliferative index compared to *SGC-7901* and *7901-zeo* cells, suggesting that transfection of *SGC-7901* cells with antisense *NHE1* gene resulted in leftward shift of the cell cycles and reduced capacity for differentiation and proliferation. Flow cytometry showed apoptotic peak of *7901-AS* cells with apoptotic rate of 26.1%, which was significantly higher than that of *SGC-7901* cells (4.5%) and *7901-zeo* cells (5.1%), suggesting that transfection with antisense *NHE1* induced apoptosis of *SGC-7901* cells (Table 2).

Tumorigenicity assay in nude mice

After inoculation with 4×10^6 *SGC-7901* cells or *7901-zeo* cells, all mice ($n = 3$) grew palpable tumors on the sixteenth-seventeenth day. Subsequently, the tumors grew progressively. The size and speed of growth of the two different tumors showed no apparent difference at 2 mo. After inoculation with 4×10^6 and 8×10^6 dose of *7901-AS* cells, none of the mice ($n = 3$, respectively) grew palpable tumors within 2 mo. This result indicates that antisense *NHE1*-transfected *SGC-7901* cells completely loose tumorigenecity in nude mice.

Table 2 Cell cycle distribution and the apoptotic rate in transfected and untransfected cells

Cell type	Cell cycle distribution (%)				Apoptotic rate
	G_0/G_1	S	G_2/M	PI	
7901-AS	60.0	34.3	6.3	40.3	26.1[b]
7901-zeo	54.6	39.0	7.6	46.6	5.1
SGC-7901	55.4	37.9	8.0	45.2	4.5

[b]$P < 0.01$ *vs* *SGC-7901* and *7901-zeo*.

DISCUSSION

Since the successful cloning of human *NHE1*cDNA in 1989 by the Sardet Laboratory[6], the 8 known subtypes of NHE, named as NHE1-8 respectively, have been shown to be identical in structure and are related in function. These form the gene family of membrane exchange protein[7-10]. The different subtypes have different number and distribution, different pharmacological properties, and are regulated by different factors. *NHE1*, a house-keeping gene, located at 1p[1] with mRNA of 3.8 Kb, is found in nearly all human tissues, and serves to remove excessive H^+ in the cytoplasm by the Na^+-H^+ exchange system, resulting in the maintenance of a stable pH_i[11-14]. Glycolysis of tumor cells may result in the production of large quantities of lactic acid and H^+. Researchers have long believed that the pH_i of tumor cells is more acidic than that of normal cells. However, ^{31}P-NMR spectroscopic studies of tumor cells have shown that the pH_i measured *in situ* is more alkaline (pH 7.0-7.2) than that of the normal cells (pH 6.5-7.0). This phenomenon of high pH_i with low extracellular pH (pH_e) is caused by the activation of NHE1 in the cell membrane with increased mRNA expression,

resulting in strong Na^+-H^+ exchange in the tumor cells. Most of the H^+ pumped out of the cells helps to create an acidic environment in the interstitial fluid of the tumor cells and maintains the pH_i in the tumor cells as neutral or more basic[15-18]. Our previous studies have shown that the significantly greater expression level of NHE1 protein in gastric carcinoma tissues compared to that in normal gastric tissues is closely associated with the genesis and progression of tumors[5], suggesting that *NHE1* can be used as the target site in the treatment of such tumors. However, further studies are needed to determine whether or not the intervention of antisense *NHE1* gene can decrease type 1 Na^+/H^+ exchanger of membranous ion exchange protein in gastric carcinoma cells and reverse the malignant phenotypes. Therefore, in the present study, the purpose of transfection of antisense *NHE1* gene into human gastric carcinoma cell line *SGC-7901* was to investigate the effects of antisense therapy targeting *NHE1* gene in the malignant phenotypes of gastric carcinoma cells. It was observed that reduced pH_i partially reversed the malignant phenotypes of *SGC-7901* cells transfected with antisense *NHE1*. Compared with *SGC-7901* and *7901-zeo* cells, the *7901-AS* cells showed cell proliferation inhibition, G_1/G_0 phase arrest, increased cell apoptotic rate, recovery of contact inhibition and density contact, decreased invasive capacity, and loss of cloning efficiency in soft agar, and tumorigenecity in nude mice. These results indicate that the *NHE1* gene is important in maintaining the phenotypes of the *SGC-7901* cell line. The *NHE1* gene may be closely associated with the malignant biological behavior of the tumor cells, and as a result, the phenotype of the tumor is restrained when the *NHE1* gene is inhibited.

Overexpression of *NHE1* gene plays an important role in the regulation of pH_i in tumor cells[19-26]. The enhancement of Na^+-H^+ exchange by tumor cells, caused by increasing the quantitative distribution of NHE1 on the cell membrane, is the major molecular mechanism in the regulation of pH_i in tumor cells. This step is of important biological significance in the maintenance of stable pH_i and malignant growth of the tumor cells. The energy consumption process of Na^+-H^+ exchange which is dependent on the energy supplied by Na^+-K^+-ATPase, stimulates glucose absorption and glycolysis by tumor cells and produces more intracellular H^+, leading to strong Na^+-H^+ exchange in the tumor cells[27-30]. Most of the H^+ pumped out of the cells helps to create the acidic environment in the interstitial fluid of the tumor cells and keeps the pH_i neutral or more alkaline in tumor cells, resulting in the protection of the tumor cells from apoptosis[18,23]. In the present study, transfection of antisense *NHE1* gene inhibited the expression of *NHE1* gene in *SGC-7901* gastric carcinoma cells, resulting in intracellular acidification and induced apoptosis of *SGC-7901* cells. The inhibited proliferation, increased apoptotic rate, and decreased malignancy in tumor cells resulted in significantly reduced tumorigenicity in nude mice *in vivo*. These results indicate that transfected human *NHE1* antisense gene successfully inhibited Na^+-H^+ exchange and destroyed the energy metabolism pattern of gastric carcinoma cells. Our findings point to the feasibility of treatment of gastric carcinoma by induction of cell apoptosis through the process of intracellular acidification, which may provide a new method for gene therapy of gastric carcinoma.

In conclusion, we transfected *NHE1* antisense into *SGC-7901* cells and observed the morphological and biological changes in the tumor cells. Our results reveal that *NHE1* antisense gene transfection can partly reverse the malignant behavior, resulting in intracellular acidification and induction of apoptosis of *SGC-7901* cells. These finding may provide a potential method for gastric carcinoma gene therapy in the future.

COMMENTS

Background

Gastric cancer is one of the most common malignant tumors in China, but the pathogenesis is unclear. Recent investigations have demonstrated that type 1 Na^+/H^+ exchanger (*NHE1*) mRNA expression is significantly increased in the carcinoma, relative to the occurrence and growth of tumors. Our previous studies have shown that significantly higher expression level of NHE1 protein in gastric carcinoma tissue compared to normal gastric tissue is closely associated with the genesis and progression of tumors. Therefore, the *NHE1* gene may be a good target for antisense gene therapy for gastric cancer. However, further studies are needed to determine whether antisense *NHE1* gene intervention can reduce type 1 Na^+/H^+ exchanger of the membranous ion exchange protein, and influence the biological behavior of the gastric carcinoma cells.

Research frontiers

Over expression of *NHE1* gene plays an important role in the regulation of pH_i in tumor cells, which is of important biological significance in the malignant growth of tumor cells. However, the role of NHE1 in the regulation of tumorigenic and metastatic properties of tumor cells remains unclear and it is important to determine the precise role of *NHE1*.

Innovations and breakthroughs

Our results reveal that *NHE1* antisense gene may significantly restrain the malignant behavior of human gastric carcinoma cells, result in intracellular acidification, suppress cell growth, induce cell apoptosis, and partially reverse the malignant phenotypes of *SGC-7901*. These findings suggest a potential role for human tumor gene therapy.

Applications

The present study may be helpful in determining a potential role for gastric carcinoma gene therapy in the future.

Terminology

The term pH_i means intracellular pH. The pH_i of tumor cells measured in situ is more alkaline than that of normal cells. This phenomenon of high pH_i with low extracellular pH (pH_e) is caused by the activation of *NHE1* in cell membrane and increased mRNA expression. *NHE1*, a type 1 Na^+/H^+ exchanger, is a transmembrane protein found in all eukaryotic cells. One of its functions is to reduce excess H^+ in the cytoplasm by means of Na^+-H^+ exchange, resulting in stable intracellular pH levels.

Peer review

This is an interesting article which examines the effect of inhibiting NHE1 on tumor survival. The manuscript is of interest and the data is good. It would be useful if NHE1 can be explained in more detail in the abstract.

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LIVER CANCER

Troglitazone, a peroxisome proliferator-activated receptor γ ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells

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Abstract

AIM: To examine the effect of troglitazone, a peroxisome proliferator-activated receptor γ (PPARγ) ligand, on the proliferation and apoptosis of human liver cancer cells.

METHODS: Liver cancer cell line HepG2 was cultured and treated with troglitazone. Cell proliferation was detected by 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay; apoptosis was detected by flow cytometry and terminal deoxynucleotidyl transferase-mediated nick end labeling of DNA fragmentation sites (TUNEL) assay; and apoptosis-related protein was detected by immunocytochemistry and Western blotting.

RESULTS: Troglitazone inhibited growth and induced apoptosis of HepG2 cells in a dose-dependent manner, and induced activation of caspase-3 expression. Troglitazone not only drove apoptosis-inhibiting factor survivin to translocate incompletely from the nucleus to the cytoplasm, but also inhibited expression of survivin, while it did not affect expression of apoptosis-promoting factor Bax.

CONCLUSION: PPARγ ligands inhibit growth and induce apoptosis of liver cancer cells, and may have applications for the prevention and treatment of liver cancer.

Key words: Peroxisome proliferator-activated receptor γ; Troglitazone; Liver neoplasms; Apoptosis

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) is the fifth most common malignant tumor worldwide, and causes more than 500 000 deaths annually[1]. Dissemination of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) causes the incidence of HCC to increase[2]. Surgical resection is the treatment of choice for HCC, but only 10%-20% of HCC patients are resectable at the time of diagnosis[3]. Liver transplantation is only indicated for early HCC (Milan criteria 1 tumor, < 5 cm in diameter, or up to three tumors, all < 3 cm in diameter), as far as outcome is concerned[4]. In addition, recurrence remains high in patients who receive radical treatment[5,6]. It is therefore urgent to seek a new approach for the treatment of HCC.

Peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor superfamily[7]. Three subtypes of PPAR (PPARα, PPARγ and PPARδ) have been identified, among which PPARγ has been studied most extensively. PPAR-γ exerts its effects by forming heterodimers with the 9-*cis*-retinoid X receptor (RXR) after ligand activation. The PPAR/RXR heterodimer acts as a regulatory transcription factor with peroxisome proliferator responsive element (PPRE) to regulate the expression of target genes which participate in the physiological and pathological processes of lipid metabolism, glucose metabolism, adipocyte differentiation, energy balance, inflammatory reactions and atherosclerosis[8,9]. PPARγ ligands are classified as natural and synthetic. The former include long-chain polyunsaturated fatty acid and eicosanoids, and the latter include thiazolidinediones (TZDs), the most commonly used drugs for diabetes, such as troglitazone, pioglitazone, rosiglitazone and LY171.833. Several recent studies have shown most human tumors

express PPARγ, and there is evidence that PPARγ ligands have anti-tumor activity[10-20]. One of the important effects of these drugs is induction of apoptosis, although the exact mechanism remains elusive.

The present study used liver cancer cell line HepG2 that endogenously expresses PPARγ as an experimental model[15] to study the effects of PPARγ ligand troglitazone on proliferation and apoptosis of liver cancer cells. It also analyzed the molecular mechanisms of these effects in an attempt to provide experimental clues for the use of PPARγ ligands in the treatment of liver cancer.

MATERIALS AND METHODS

Chemicals

Troglitazone (Cayman Chemical Industry, USA) was dissolved in DMSO and then diluted to appropriate concentrations with culture medium. The final concentration of DMSO in the culture medium did not exceed 1 mL/L.

Cell culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, GIBCO Laboratories), 100 U/mL penicillin, and 100 g/mL streptomycin. Cells were grown at 37℃ in an atmosphere of 95% air and 5% CO_2.

MTT assay

Cell proliferation was detected by 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) assay. When cells were cultured to the log phase, they were seeded on a 96-well plate (2×10^4 cells/100 μL/well) for 24 h until confluence. Cells were divided into a control (DMSO) group and a troglitazone group. The concentration of troglitazone was 5, 10, 20, 40, 80 and 100 μmol/L. After 120 h, 10 μL MTT (5 g/L) was added and incubated at 37℃ for 4 h. DMSO (75 μL) was added to each well, which was oscillated for 10 min until the crystals were dissolved completely. Absorbance (A) was detected with an enzyme calibrator at 560 nm. Cell viability = (A of study group/A of control group) × 100%. The experiment was repeated twice. There were six wells for each concentration.

Flow cytometry

Cells were seeded onto a six-well plate and allowed to grow to 40% confluence. After treatment with or without the drug, both floating and adherent cells were collected. Cells were suspended and then fixed with ice-cold 70% alcohol at -20℃, followed by washing and staining with 50 μg/mL propidium iodide in the presence of 100 μg/mL ribonuclease A for 30 min at 37℃ in the dark. DNA content was analyzed by flow cytometry using Cell Quest software (Becton Dickinson and Beckman-Coulter, San Jose, CA, USA).

TUNEL assay

HepG2 cells were grown on poly-L-lysine-coated slides in a six-well plate. After treatment with or without the drug, the slides were gently washed three times in 0.1 mol/L PBS (pH 7.4), fixed with 80% ice-cold ethanol, and immediately transferred to a freezer until use. To study apoptosis of cultured cells, terminal deoxynucleotidyl transferase-mediated nick end labeling of DNA fragmentation sites (TUNEL) assay was performed using Fluorescein FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, San Diego, CA, USA) according to the manufacturer's instructions. Ten high-power areas with even distribution of positive cells were used for calculation of the percentage of apoptotic cells under a fluorescence microscope.

Immunocytochemistry

HepG2 cells were grown on poly-L-lysine-coated slides in a six-well plate. After treatment with the drug, the slides were washed twice with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Immunostaining was performed using the streptavidin-biotin complex method with UltraSensitive S-P Kit (Fuzhou Maxim Biotech, Fuzhou, Fujian, China). The slides were pretreated first with 0.3% hydrogen peroxide in PBS for 10 min to inactivate endogenous peroxidase, and then microwave antigen retrieval was performed with 0.01 mol/L citrate buffer at pH 6.0 for 20 min, followed by incubation with polyclonal rabbit anti-human antibody against the active caspase-3 (1:25; R&D Systems, Wiesbaden, Germany), rabbit anti-human survivin polyclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4℃ overnight, biotinylated secondary antibody for 30 min, and peroxidase-labeled streptavidin for 30 min. Color reaction was developed with 3, 3'-diaminobenzidine as a chromogen. Finally, the slides were counterstained with hematoxylin, and dehydrated through graded alcohol. For negative controls, slides were processed as above but treated with PBS instead of the primary antibody.

Western blotting

Troglitazone-treated HepG2 cells were prepared in a cell-lysis solution. The protein concentration was determined by BCA Protein assay kit (Perbio Science Deutschland, Germany). Protein samples (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes, to which rabbit anti-human survivin polyclonal antibody (1:1000), mouse anti-human Bax antibody (1:1000), mouse anti-human β-actin antibody (1:1000), and horseradish-peroxidase-labeled secondary antibody (all from Santa Cruz Biotechnology) were added. The signals were detected by enhanced chemiluminescence according to the protocol supplied with the kit.

Statistical analysis

Data were expressed as mean ± SD. Statistical correlation of data was checked for significance by ANOVA and Student's *t* test. Differences with $P < 0.05$ were considered significant. These analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL, USA).

RESULTS

Troglitazone inhibits cell growth

The MTT assay showed that cell viability was 96.8% ± 1.22%,

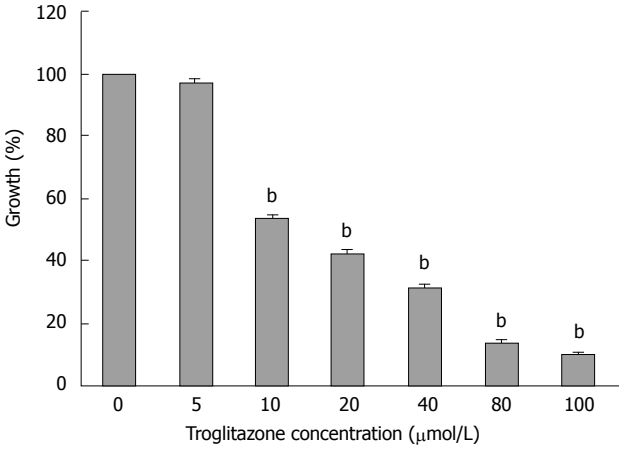

Figure 1 Effect of troglitazone on growth of HepG2 cells. Cells were incubated with different concentrations of troglitazone for 120 h. Troglitazone inhibited growth of HepG2 cells in a dose-dependent manner, as demonstrated by MTT assay. Data represent the mean ± SD from six wells. $^{b}P < 0.01$ *vs* corresponding control group (unpaired Student's *t* test).

53.4% ± 1.2%, 42.3% ± 1.2%, 31.4% ± 1%, 13.6% ± 0.8% and 9.6% ± 0.7% at 5, 10, 20, 40, 80 and 100 μmol/L troglitazone for 120 h, respectively (Figure 1), which indicated that troglitazone inhibited the growth of liver cells in a dose-dependent manner.

Troglitazone induces apoptosis

HepG2 cells were treated with troglitazone (20 and 30 μmol/L) for 24 h, and phase-contrast microscopy revealed that some cells became round, blunt and smaller in size, accompanied by karyopyknosis; light refraction was increased; and cells became detached and suspended in the medium, especially with 30 μmol/L troglitazone. In the control group, cells were regular in morphology and grew fully in patches and confluently, rarely sloughing off (Figure 2).

Flow cytometry showed that after treatment of HepG2 cells with 20 and 30 μmol/L troglitazone for 24 h, the percentage of sub-G_0/G_1 peak apoptotic cells was 25.5% ± 1.1% and 43.9% ± 1.7%, respectively. In the untreated controls, none of the cells was found in the sub-G_0/G_1 peak (Figure 3). TUNEL staining also showed that treatment with troglitazone for 24 h caused apoptosis of HepG2 cells. The apoptotic rate increased with concentration of troglitazone: 22.4% ± 1.2% for 20 μmol/L, and 38.6% ± 0.8% for 30 μmol/L troglitazone. In the control group, there were only background cells, without the presence of TUNEL positive cells (Figure 4, $P < 0.001$).

Troglitazone induces activation of caspase-3 expression

The caspase family plays a very important role in mediating apoptosis, among which caspase-3 is the key executive molecule, and its activated form has multiple functions in signal transmission of apoptosis[21,22]. Troglitazone activated caspase-3 expression. Positive staining was signified by a yellow-brown color, present in smaller cells whose plasma was condensed. After treatment of HepG2 cells with 20 and 30 μmol/L troglitazone, the positive cell rate was 21.2% ± 1.4% and 35.8% ± 2.4%, respectively (Figure 5). No positive cells were found in the controls.

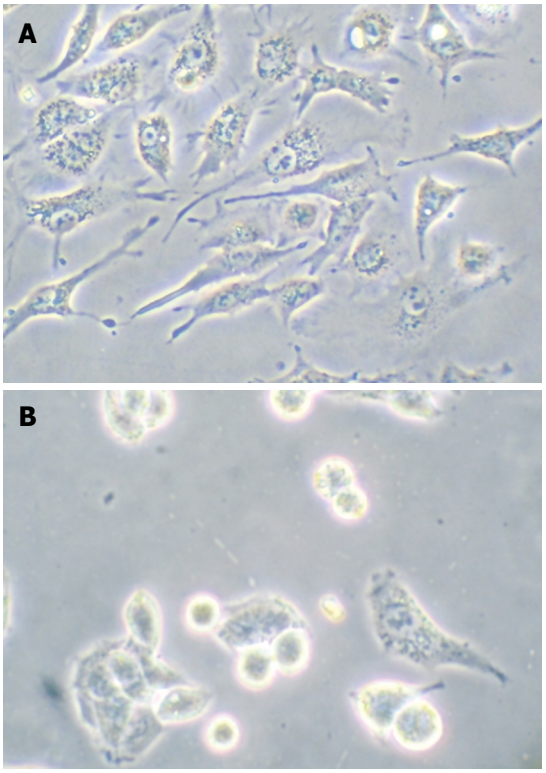

Figure 2 Morphological changes were examined by phase-contrast microscopy. **A**: Controls; **B**: 30 μmol/L troglitazone.

Troglitazone drives survivin protein to translocate incompletely from the nucleus to the cytoplasm and inhibits its expression

Immunocytochemical staining showed that apoptosis-inhibiting factor survivin was mainly expressed in the nucleus and weakly in the cytoplasm of untreated HepG2 cells. After treatment with 20 and 30 μmol/L troglitazone for 24 h, survivin translocated partly from the nucleus to the cytoplasm, and the intensity of its expression decreased markedly. Also, apoptotic cells were seen in the treatment group, and presented with a smaller cell size and condensed cytoplasm (Figure 6). Western blotting revealed that, in HepG2 cells treated with troglitazone for 24 h, expression of HepG2 survivin was inhibited in a dose-dependent manner, but the treatment did not significantly affect expression of apoptosis-promoting factor Bax (Figure 7).

DISCUSSION

Apoptosis is a normal phenomenon in the process of embryonic growth of all organisms and human development. Disturbance of this process is associated with the development of many severe diseases and disorders including tumors. Selective induction of tumor-cell apoptosis may become the basic strategy in the treatment of malignant tumors. Most of the available chemotherapy drugs work through destroying tumor cells by inducing apoptosis[23]. In our study, the MTT assay showed troglitazone inhibited growth of liver cancer cells in a dose-dependent manner. To determine whether the inhibitory effect of troglitazone on the proliferation of liver cancer cells was associated with induction of apoptosis, flow cytometry and TUNEL

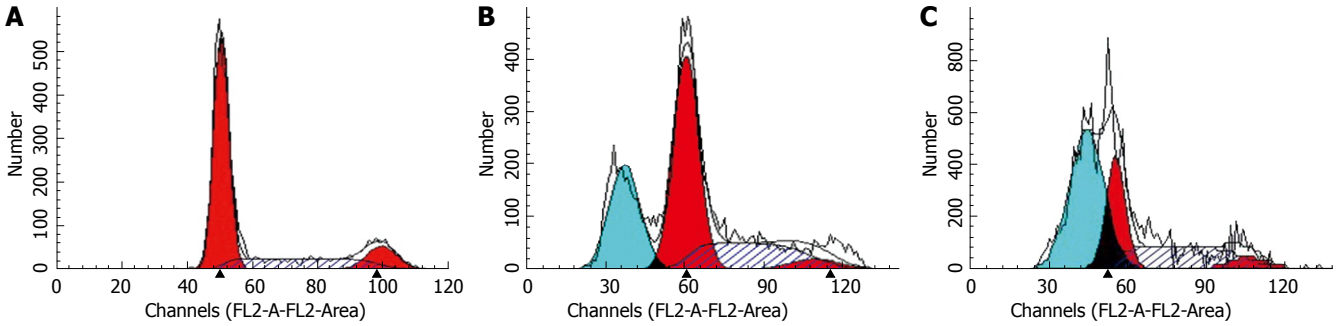

Figure 3 Cell cycle analysis by flow cytometry. **A**: Control cells; **B**: 20 μmol/L troglitazone; **C**: 30 μmol/L troglitazone.

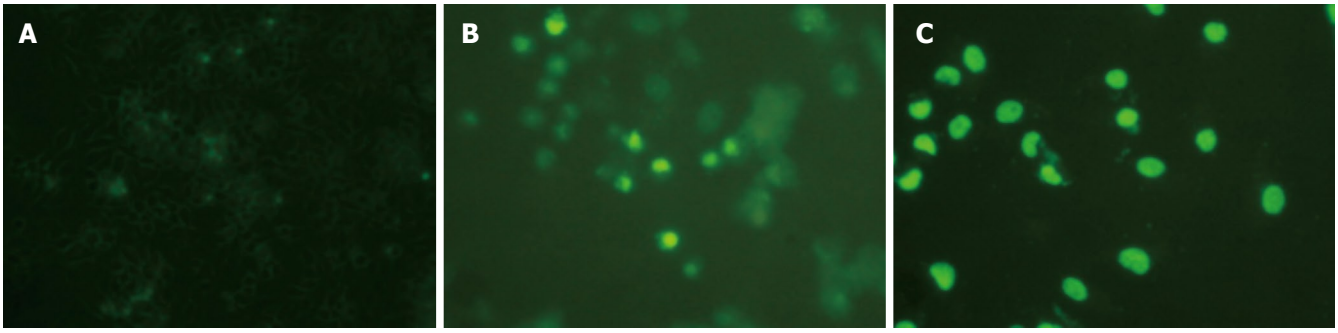

Figure 4 Troglitazone significantly increased the number of TUNEL-positive cells in a dose-dependent manner. **A**: Control cells; **B**: 20 μmol/L troglitazone; **C**: 30 μmol/L troglitazone.

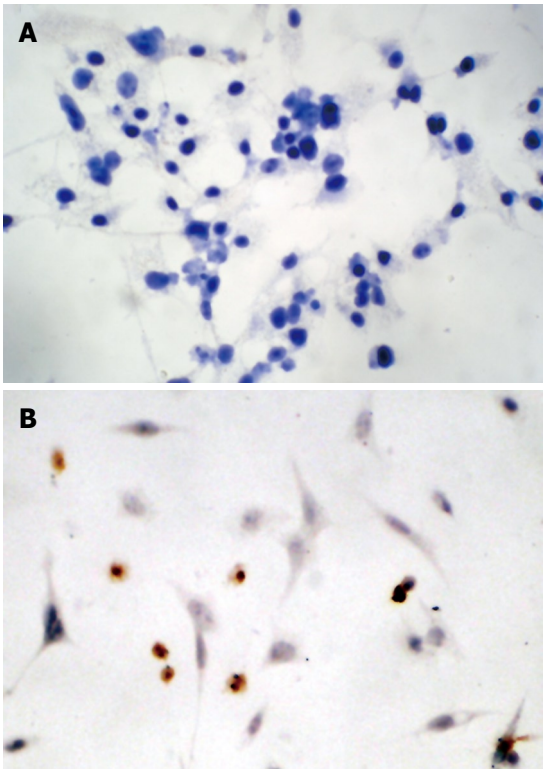

Figure 5 Immunocytochemistry showed that caspase-3 was activated. The shrunken cells were positively stained for activated caspase-3. No positive cell was detectable in the control group (**A**: original magnification, × 200). Whereas large numbers of positive cells labeled for activated caspase-3 were observed in HepG2 cells treated with 30 μmol/L troglitazone for 24 h (**B**: original magnification, × 400).

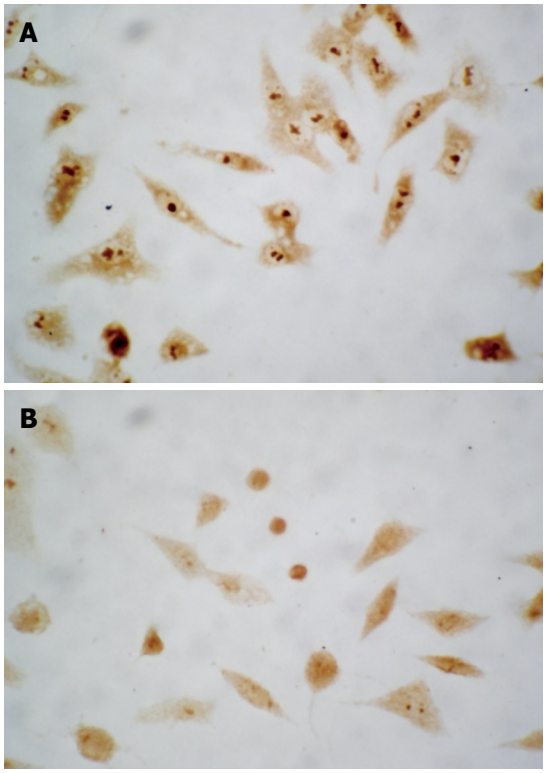

Figure 6 Survivin was present predominantly in the nucleus in untreated cells (**A**: original magnification, × 400) as demonstrated by immunocytochemistry. After exposure of HepG2 cells to 30 μmol/L troglitazone for 24 h, survivin incompletely translocated from the nucleus to the cytoplasma (**B**: original magnification, × 400).

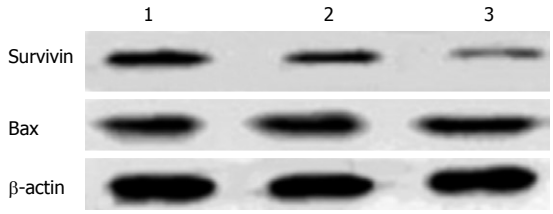

Figure 7 Effect of troglitazone on the expression of Bax and survivin. Lane 1: Control cells; lane 2: 20 μmol/L troglitazone; lane 3: 30 μmol/L troglitazone.

staining were conducted. The results confirmed troglitazone induced apoptosis of HepG2 cells in a dose-dependent manner. Thus, clarifying the apoptosis-related gene protein that is associated with troglitazone-induced apoptosis is of primary importance in explaining the mechanism of action of troglitazone.

Caspase is a group of cysteine hydrolytic proteinases that are able to specifically cleave the peptide chain behind the residue base of the target protein aspartate. It is the key molecule in the regulation of apoptosis. Caspase can trigger a cascade reaction under the control of apoptosis signals. According to their method of entering the apoptotic site, caspases are classified as initiator and effector caspases[21], among which, caspase-3 is the most important terminal effector caspase in apoptosis, and plays an irreplaceable role. After initiation of the apoptotic process, caspase-3 transforms from the zymogen form to the activated form and functions by hydrolyzing proteins essential for survival of many kinds of cells[22].

Survivin is a member of the inhibitor of apoptosis (IAP) family of negative regulators of programmed cell death, and is undetectable in normal adult tissue, but is abundantly expressed in fetal tissue and a variety of human cancers including HCC. High levels of survivin in tumors are frequently correlated with malignant parameters, which suggest a role in tumorigenesis[24-26]. Survivin can inhibit terminal effector caspase-3 and caspase-7, and interfere with caspase-9 activity and processing. Therefore, approaches designed to counteract antiapoptotic activity may inhibit tumor growth[27].

There has long been controversy over the subcellular location of survivin. Some researchers have found survivin expression is located in the nucleus in HCC, esophageal squamous cell carcinoma, ovarian carcinoma, mantle cell lymphoma, cholangiocarcinoma, and non-small cell lung cancer, and is associated with poor prognosis. Other researchers have pointed out that survivin expression is located in the nucleus in gastric cancer, bladder mucosa and transitional cell carcinoma, and breast cancer, and is a predictor of good prognosis[28]. The results of our immunohistochemical staining confirmed that survivin was expressed mainly in the nucleus of HepG2 cells, and weakly in the cytoplasm. Fortugno *et al*[29] have found two survivin pools using immunofluorescence, one in the cytoplasm and the other in the nucleus. Suzuki *et al*[30] have found survivin protein can translocate from the cytoplasm to the nucleus. Here, it forms a complex with CDK4, which enables p21 to combine with a precursor released from the CDK4 complex and mitochondrial caspase-3, and inhibits apoptosis by inhibiting the activity of caspase-3. The results of our study showed troglitazone induced apoptosis in a dose-dependent manner, during which survivin translocated incompletely from the nucleus to the cytoplasm, with a decrease in the intensity of expression; at the same time caspase-3 expression was activated. We presume troglitazone dissociated the survivin/CDK4 complex by inducing survivin expression and driving survivin to translocate incompletely from the nucleus to the cytoplasm. This forced p21 to form a complex with CDK4 by dissociating the combination of p21 and the precursor of mitochondrial caspase-3 and activating caspase-3. This led to cleavage of the corresponding nuclear and cytoplasmic substrates and finally caused apoptosis.

TZD induces massive apoptosis in renal cancer cells, with increased Bax expression and decreased Bcl-2 expression[18]. However, troglitazone significantly increases the expression of c-myc mRNA, but has no effect on expression of Bcl-2 and Bax in thyroid carcinoma cells[13]. In the current study, we also found Bax may not participate in troglitazone-induced apoptosis, suggesting that the mechanisms by which the PPARγ ligands cause tumor cell apoptosis are different depending on the type of cancer.

In conclusion, PPARγ ligands have the effect of inhibiting growth and inducing the apoptosis of liver cancer cells, and may have applications for the prevention and treatment of liver cancer.

COMMENTS

Background

Peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor superfamily. Three subtypes of PPAR (PPARα, PPARγ and PPARδ) have been identified, among which PPARγ has been studied most extensively. Recent data have shown that ligands for PPARγ exhibit growth-inhibitory effects on many types of human cancer. One of the important effects of these drugs is inducing apoptosis, although the exact mechanism remains elusive.

Research frontiers

We used liver cancer cell line HepG2 that endogenously expresses PPARγ as an experimental model to study the effects of PPARγ ligand troglitazone on the proliferation and apoptosis of liver cancer cells, and analyzed the molecular mechanisms of these effects.

Innovations and breakthroughs

Troglitazone inhibited growth and induced apoptosis of HepG2 cells in a dose-dependent manner, and induced activation of caspase-3 expression. Troglitazone not only drove apoptosis-inhibiting factor survivin to translocate incompletely from the nucleus to the cytoplasm, but also inhibited expression of survivin.

Applications

PPARγ ligand has the effect of inhibiting growth and inducing apoptosis of liver cancer cells, and may have potential as a drug against liver cancer.

Peer review

This study should arouse interest in readers. It provides important information for the investigation of the anti-cancer mechanism of troglitazone and other PPARγ ligands, and for the development of anti-cancer therapy using PPARγ ligands.

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BASIC RESEARCH

Detection of apoptosis induced by new type gosling viral enteritis virus *in vitro* through fluorescein annexin V-FITC/PI double labeling

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Abstract

AIM: To achieve a better understanding of the pathogenesis of new type gosling viral enteritis virus (NGVEV) and the relationship between NGVEV and host cells.

METHODS: The apoptosis of duck embryo fibroblasts (DEF) induced by NGVEV was investigated by fluorescence-activated cell sorter (FACS) and fluorescence microscope after the cells were stained with Annexin V-FITC and propidium iodide (PI).

RESULTS: By staining cells with a combination of fluorescein annexin V-FITC and PI, it is possible to distinguish and quantitatively analyze non-apoptotic cells (Annexin V-FITC negative/PI negative), early apoptotic cells (Annexin V-FITC positive/PI negative), late apoptotic/necrotic cells (Annexin V-FITC positive/PI positive) and dead cells (Annexin V-FITC negative/PI positive) through flow cytometry and fluorescence microscope. The percentage of apoptotic cells increased with the incubation time and reached a maximum at 120 h after infection, while the percentage of non-apoptotic cells decreased.

CONCLUSION: NGVEV can induce the infected DEF cells to undergo apoptosis and the apoptosis occurs prior to necrosis.

Key words: Gosling viral enteritis; New type; Virus; Duck embryo fibroblasts; Apoptosis; Fluorescein annexin V-FITC/PI

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INTRODUCTION

In order to eliminate the redundant, damaged, or infected cells, metazoan organisms evolve the cell suicide mechanism termed apoptosis[1]. Apoptosis is a physiological process defined by a number of distinct morphological features and biochemical processes[2,3], which distinguish from necrosis[4,5]. Apoptosis is recognized as an important process in different biological systems, including embryonic development, cell turnover, and immune response against tumorigenic or virus-infected cells[6-8]. An increasing number of viruses or viral gene products were reported to induce apoptosis both *in vitro* and *in vivo*[9-16].

The new type gosling viral enteritis (NGVE) is a new infectious disease and firstly recognized by Cheng *et al*, and it was observed in goslings less than 30 d of age in various areas of Sichuan Province[17,18]. The mortality from acute NGVE is high, and it is clinically characterized by respiratory, digestive, and neurological symptoms and

sudden death[17-19]. Catarrhal hemorrhagic fibrinonecrotic enteritis of the small intestine and coagulative embolus in the lower middle part of the intestine are the typical pathological changes of the NGVE in infected goslings[18]. NGVE virus was recognized as an adenovirus, which was round or oval, and characteristic icosahedral in shape, containing double-stranded DNA genome and fifteen structural proteins[18-20]. There are many researches on the histopathology, epizootiology, clinical signs, diagnoses, and immunity of the NGVE[17-24]. Interestingly, the apoptosis induced by NGVE virus infection is poorly documented.

In the early stage of apoptosis, which occurs at the cell surface, one of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell[25-27]. Annexin V-FITC is a phospholipid-binding protein with a high affinity for PS, which can be used as a sensitive probe for PS exposure to the cell membrane[28-30]. However, it has also been reported that it binds to the inner face of the plasma membrane that has lost its integrity during the late stage of apoptosis, also known as secondary necrosis[31]. During the early apoptosis, the cells become reactive with annexin V-FITC after the onset of chromatin condensation, but prior to the loss of the plasma membrane ability to exclude PI[32]. Hence, necrotic cells are both stained by annexin V-FITC and PI, whereas early apoptotic cells are only stained by annexin V-FITC. Double staining of the infected DEF cells with annexin V-FITC and PI in this research could distinguish apoptotic cells from necrotic cells[33]. In this way, live, early apoptotic, late apoptotic/necrotic and dead cells can be discriminated on the basis of a double-labeling for annexin V-FITC and PI, and analyzed by either flow cytometry or fluorescence microscopy[34,35].

MATERIALS AND METHODS

Primary duck embryo fibroblast (DEF) and viral strain

DEF cells were prepared using 11 to 13-d-old embryonated specific pathogen-free (SPF) eggs and propagated in minimal essential medium (MEM; Gibco) containing 100 mL/L new born calf serum (NBCS; Hyclone), 2.2 g/L $NaHCO_3$, 100 U/mL penicillin/streptomycin (Gibco).

The NGVEV-CN strain with a high virulence field was provided by the Avian Diseases Research Centre of the Sichuan Agricultural University. The initial strain (adapted for cell culture growth) was isolated from a natural NGVE virus infection and the SPF gosling was then artificially infected, and the virus was serially passaged in 10-day-old SPF embryo eggs. The allantoic fluid was harvested and adapted to the monolayer DEF cells.

Experimental NGVE virus infection of DEF

The monolayer DEF cells were washed twice with phosphate buffered saline solution (PBS; 0.15 mol/L, pH 7.2) and subsequently exposed to stock NGVEV-CN on a shaker at 37.5℃ for 2 h. Stock virus was harvested from infected DEF when 75% cytopathic effects (CPEs) were observed. After inoculation with NGVEV-CN, cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2 in MEM supplemented with penicillin/streptomycin and 20 mL/L NBCS. Mock-infected cells were processed in the same way except that the virus was excluded.

Table 1 Apoptotic rate of DEF cells detected through annexin V-FITC/PI staining and analyzed by FACS

Infection time (h)	Early apoptotic cells (%) (annexin V-FITC$^+$/PI$^-$)		Advanced apoptotic cells (%) (annexin V-FITC$^+$/PI$^+$)	
	Mock infected	NGVEV infected	Mock infected	NGVEV infected
24	3.6	4.3	0.3	0.3
48	3.9	8.8	0.8	1.3
72	4.6	21.5	1.7	1.9
96	5.2	30.8	3.4	7.8
120	6.1	35.3	4.5	13.9
144	7.2	33.7	5.1	17.7

Annexin V-FITC/PI stained fluorescence-activated cell sorter (FACS)

At 24, 48, 72, 96, 120 and 144 h after infection (p.i.), 3 infected and mock-infected cells were harvested through trypsinization, and washed twice with cold PBS (0.15 mol/L, pH 7.2). The cells were centrifuged at 3000 r/min for 5 min, then the supernatant was discarded and the pellet was resuspended in 1 × binding buffer at a density of 1.0×10^5-1.0×10^6 cells per mL. One hundred μL of the sample solution was transferred to a 5 mL culture tube, and incubated with 5 μL of FITC-conjugated annexin V (Pharmingen) and 5 μL of PI (Pharmingen) for 15 min at room temperature in the dark. Four hundred μL of 1 × binding buffer was added to each sample tube, and the samples were analyzed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson).

Annexin V-FITC/PI stained fluorescence microscopy

The annexin V-FITC/PI staining procedure of the sample was adopted as above except that 1.0×10^6 cells/mL were centrifuged onto glass slides and studied under fluorescence microscope (Nikon 80i).

RESULTS

Annexin V-FITC/PI stained FACS

By staining cells with annexin V-FITC and PI, FACS was used to distinguish and quantitatively determine the percentage of dead, viable, apoptotic and necrotic cells after NGVE virus infection (Figure 1 and Table 1). At 72 h p.i., the percentage of apoptotic cells increased from 4.6% in the mock-infected control culture to 21.5% (Figure 2). The percentage of early apoptotic cells increased with incubated time until 120 h p.i. reaching the maximum 35.3%, and the proportion of the late apoptotic/necrotic cells increased from 0.3% to 17.7% (Table 1). A high level of the early apoptosis was detected from 72 h p.i. and high level of the late apoptosis/necrosis was detected after 96 h p.i., while the basal level of apoptosis and necrosis was shown in the mock-infected controls (Table 1).

Annexin V-FITC/PI stained fluorescence microscopy

When examined under fluorescence microscopy, different

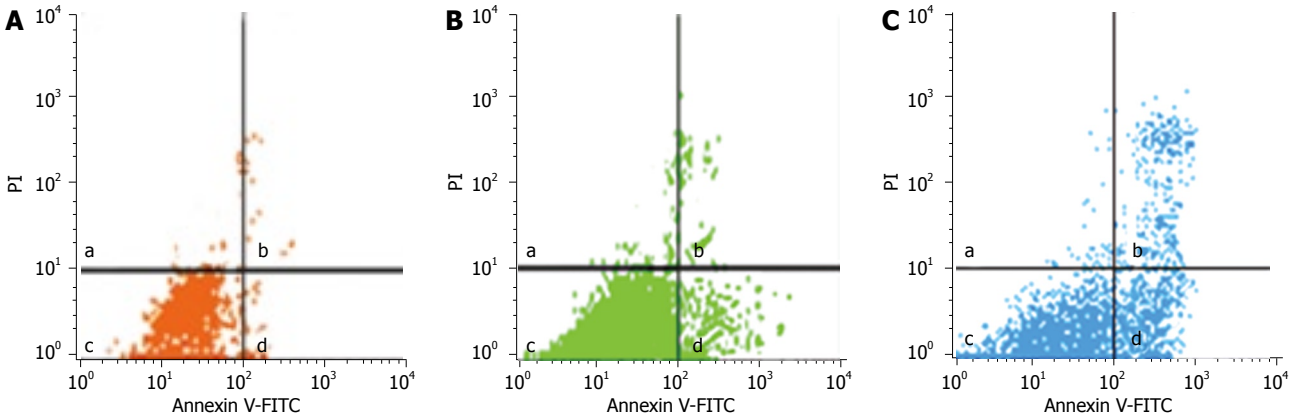

Figure 1 NGVE virus infected DEF cells analyzed by FACS, stained with annexin V-FITC/PI. (**A-C**) display the results of the cells at 48, 96 and 144 h after NGVE virus infection. The proportion of non-apoptotic cells (c: Annexin V-FITC$^-$/PI$^-$), early apoptotic cells (d: Annexin V-FITC$^+$/PI$^-$), late apoptotic/necrotic cells (b: Annexin V-FITC$^+$/PI$^+$) and dead cells (a: Annexin V-FITC$^-$/PI$^+$).

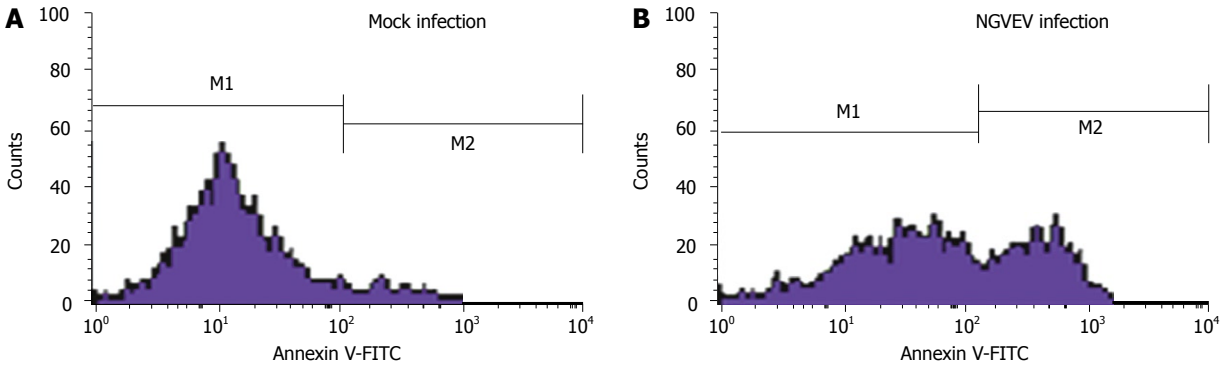

Figure 2 Flow cytometry of apoptotic DEF cells as assessed by annexin V-FITC fluorescent intensity. DEF cells are mock infected (**A**) and infected with NGVE virus (**B**). Cells harvested at 72 h p.i., and subsequently stained with annexin V-FITC/PI. One million cells are analyzed by flow cytometry, data are presented as fluorescent intensity units of annexin V-FITC (abscissa) and number of counts cells (ordinate). The M1 and M2 gates demarcate annexin V-FITC negative populations (non-apoptotic cells) and positive (apoptotic cells) populations.

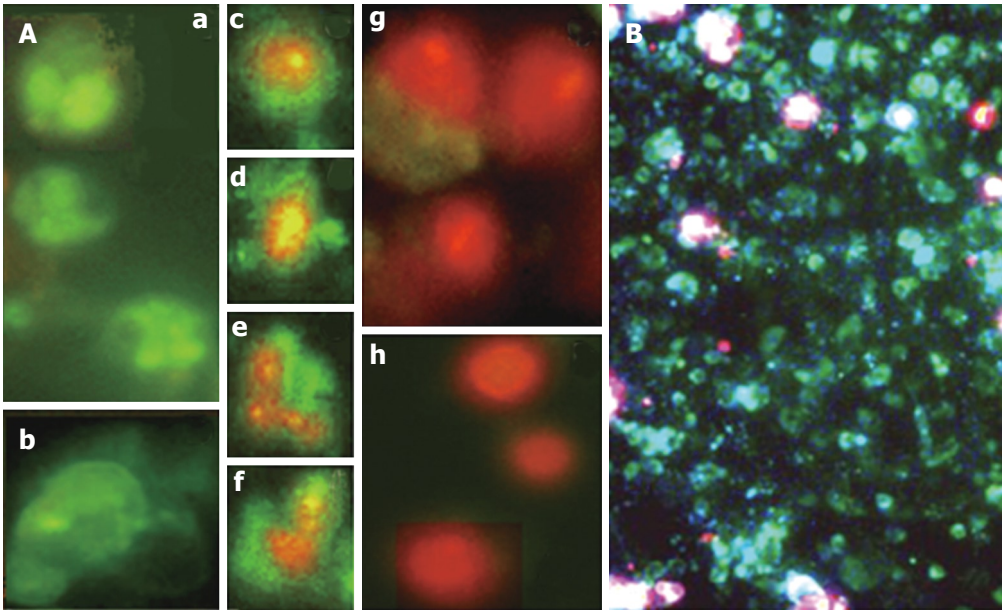

Figure 3 Apoptotic DEF cells induced by NGVE virus infection stained with Annexin V-FITC/PI and observed under fluorescence microscope. The samples are analyzed for green fluorescence (FITC) and red fluorescence (PI). **A**: Different labeling patterns of the NGVE virus infected cells: early apoptotic cells, annexin V-FITC positive and PI negative (**a** and **b**); necrotic or late apoptotic cells, both annexin V-FITC and PI positive (**c**-**f**); dead cells, annexin V-FITC negative and PI positive (**g** and **h**); **B**: 72 h p.i., the early and late apoptotic cells.

labeling patterns in this assay enabled us to identify different cell populations: live cells (Annexin V-FITC negative/PI negative), early apoptotic cells (the intactness of the cell membrane, affinity for annexin V-FITC and devoid of PI staining) (Figure 3A a, b, 3B), late apoptotic/necrotic cells (the cell membrane looses its integrity,

the cell becomes both annexin V-FITC and PI staining) (Figure 3A c-f, 3B) and dead cells (Annexin V-FITC negative/PI positive) (Figure 3A g, h, 3B).

DISCUSSION

Modulation of apoptosis is a common feature of infection by animal viruses and it also contributes to the pathogenesis process[36]. A variety of animal viruses have been identified to induce apoptosis in cultured cells[12,14-16], which contained adenovirus. Early in 1968, Takemori[37] found that *cyt* mutants of human adenovirus could provoke more violent CPEs. Ezoe[38] further proved that it could also induce the DNA degradation in infected cells. Rautenschlein[39,40] respectively reported that the hemorrhagic enteritis virus (HEV) (fowl adenovirus) could induce B cells and spleen cells undergoing apoptosis. This research indicated that NGVE virus recognized as an adenovirus[17,19,20] could induce DEF undergoing apoptosis, which has never been reported before.

FACS is frequently used to monitor early apoptosis[26-29], which should always be confirmed by the inspection of cells under electron or fluorescence microscope. Annexin V-FITC positive cells were first observed in NGVEV-infected DEF cells at 72 h p.i. under fluorescence microscopy, while it can be detected early from 24 h p.i. through FACS. The small number of apoptotic cells presented in mock-infected controls, which may be attributed to physiological cell death *in vitro*. The cells stained by annexin V-FITC alone obviously increased from 72 h p.i., indicating the induction of apoptosis rather than necrosis due to NGVE virus infection. The cells that stained positive for both annexin V-FITC and PI were increased from 96 h p.i. indicated the end stage of apoptosis or necrosis, which also suggested that apoptosis occurs prior to necrosis. This may be due to the fact that apoptosis makes many cell remnants undisturbed *in vitro*, which can be removed by phagocytes *in vivo*. The apoptotic cell debris interfered with the adjacent normal cells, leading to the necrosis. Furthermore, the lysis that eventually occurred at the end of apoptosis, which had essentially the same membrane permeability that occurred in necrosis. Further experiments are needed for a definite the intracellular events that trigger the apoptotic response during NGVE virus infection.

Recent studies demonstrate that the CPEs caused by virus infection *in vitro* is mediated by apoptosis[41-43]. Our previous research had revealed that the CPEs became obvious after 72 h p.i.[24] and $TCID_{50}$ reached a maximum at 120 h p.i., which was consistent to the results of this research: apoptotic cells obviously increased from 72 h p.i. and the apoptotic peak reached at 120 h p.i.. Therefore, it seems likely that apoptosis is related to CPEs during NGVE virus infection.

Virus-induced apoptosis is a complex and important aspect of the pathogenesis of viral infection[44-46]. In fact, in the case of virus-infected cells, the induction of cell death can reduce viral spread in the host by early killing of infected cells. In the case of virus itself, apoptosis facilitates persistent viral infection in host cells and is convenient for viral dissemination[47-49]. Quantitative assay of the apoptosis in the present study indicated that the apoptosis was largely induced in the late phase of NGVE virus infection. During late NGVE virus infection, the virus almost completes its replication, therefore, the apoptosis provided a means for releasing the virus particles into the extracellular space without initiating a concomitant host response. It is assumed that NGVE virus induction of apoptosis may be an important mechanism of the efficient dissemination of progeny and the suicide of virally infected cells through apoptosis can limit infection, affording the host organism a certain degree of protection.

Many questions regarding NGVE virus-induced apoptosis remain unanswered, and future studies should be carried out.

COMMENTS

Background

New type gosling viral enteritis (NGVE) is a new infectious disease and it is observed in goslings aged less than 30 d. The typical pathological changes of the NGVE in infected goslings are catarrhal hemorrhagic fibrinonecrotic enteritis of the small intestine and coagulative embolus in the lower middle part of the intestine. NGVE virus is recognized as an adenovirus and many reports indicated that adenovirus could induce apoptosis both *in vitro* and *in vivo*. To date, whether the NGVE virus could trigger the host cells to undergo apoptosis has not been reported.

Research frontiers

A number of viruses or viral gene products have been reported to induce apoptosis both *in vitro* and *in vivo*. Modulation of apoptosis is a common feature of infection by animal viruses and it was proved to contribute to the pathogenesis process. Scant information has been available so far for NGVE, especially in its etiology and pathogenesis.The apoptosis detected in this research during NGVE virus infection may be responsible for its pathogenesis.

Innovations and breakthroughs

The authors of this paper have indicated that, for the first time, NGVE virus could induce the apoptosis of host cells *in vitro* and the apoptosis occurs prior to necrosis. The combined use of the fluorochrome labeled with fluorescence-activated cell sorter and fluorescence microscope for apoptosis detection can provide a rapid, quantitative and objective assay of cell viability, which may be applied for enumeration of apoptotic or necrotic cells.

Applications

This work succeeded in a better understanding of pathogenesis process during NGVE virus infection.

Terminology

Apoptosis: also named as programmed cell death (PCD), is the process whereby individual cells of multicellular organisms undergo systematic self-destruction in response to a wide variety of stimuli. Apoptosis is a genetically controlled preprogrammed event which eliminates cell development when they have become redundant, or functions as an emergency response after radiation damage, viral infection, or aberrant growth induced by the activation of oncogenes.

Peer review

This is a very interesting study. The authors demonstrated that NGVE virus induces the apoptosis of infected DEF cells and the apoptosis occurs prior to necrosis. The study is well designed, and the analysis is reasonable.

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BASIC RESEARCH

Pharmacokinetics and tissue distribution of intraperitoneal 5-fluorouracil with a novel carrier solution in rats

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Abstract

AIM: To compare the pharmacokinetics and tissue distribution of 5-fluorouracil administered intraperitoneally with two isotonic carrier solutions: HAES-steri (neotype 6% hydroxyethyl starch), a novel carrier solution with middle molecular weight and physiologic saline (0.9% sodium chloride solution), a traditional carrier solution for intraperitoneal chemotherapy, in rats.

METHODS: A total of 60 Sprague Dawley rats were randomized into groups according to the carrier solution administered. Each group was further randomized according to the intraperitoneal dwell period (1, 3, 6, 12, 18 and 24 h). At the end of the procedure the rats were killed, the peritoneal fluid was withdrawn completely and quantitated. Drug concentrations in peritoneal fluid, plasma, and tissues were determined by high-performance liquid chromatography.

RESULTS: The mean volumes remaining in the peritoneal cavity were significantly higher with HAES-steri than those with physiologic saline at 1, 6, 12, 18, and 24 h (P = 0.047, 0.009, 0.005, 0.005 and 0.005 respectively, the percentages of remaining peritoneal fluid volume were 89.9 ± 5.6 *vs* 83.4 ± 4.9, 79.9 ± 2.8 *vs* 56.2 ± 15.7, 46.8 ± 5.5 *vs* 24.7 ± 9.7, 23.0 ± 2.8 *vs* 0.0 ± 0.0 and 4.2 ± 1.7 *vs* 0.0 ± 0.0 respectively). Mean concentrations in peritoneal fluid were significantly higher with HAES-steri than those with physiologic saline at 3, 12 and 18 h (P = 0.009, 0.009 and 0.005 respectively, the concentrations were 139.2768 ± 28.2317 mg/L *vs* mg/L, 11.5427 ± 3.0976 mg/L *vs* 0.0000 ± 0.0000 mg/L and 4.7724 ± 1.0936 mg/L *vs* 0.0000 ± 0.0000 mg/L respectively). Mean plasma 5-fluorouracil concentrations in portal vein were significantly higher with HAES-steri at 3, 12, 18 and 24 h (P = 0.009, 0.034, 0.005 and 0.019 respectively, the concentrations were 3.3572 ± 0.8128 mg/L *vs* 0.8794 ± 0.2394 mg/L, 0.6203 ± 0.9935 mg/L *vs* 0.0112 ± 0.0250 mg/L, 0.3725 ± 0.3871 mg/L *vs* 0.0000 ± 0.0000 mg/L, and 0.2469 ± 0.1457 mg/L *vs* 0.0000 ± 0.0000 mg/L respectively), but significantly lower at 1 h (P = 0.009, the concentrations were 4.1957 ± 0.6952 mg/L *vs* 7.7406 ± 1.2377 mg/L). There were no significant differences in the plasma 5-fluorouracil in inferior caval vein at each time-point. 5-fluorouracil concentrations were significantly greater with HAES-steri at 18 h in gastric tissue (P = 0.016, the concentrations were 0.9486 ± 0.8173 mg/L *vs* 030392 ± 0.0316 mg/L), at 18 h in colon (P = 0.009, the concentrations were 0.1730 ± 0.0446 mg/L *vs* 0.0626 ± 0.0425 mg/L), at 3, 6, 12 and 24 h in liver (P = 0.009, 0.013, 0.034 and 0.013 respectively, the concentrations were 0.6472685 ± 0.5256 mg/L *vs* 0.1554 ± 0.1043mg/L, 0.8606826 ± 0.7155 mg/L *vs* 0.0014 ± 0.0029 mg/L, 0.0445 ± 0.0330 mg/L *vs* 0.0797 ± 0.1005 mg/L and 0.0863 ± 0.0399 mg/L *vs* 0.0034 ± 0.0075 mg/L respectively) and at 18 h in lung (P = 0.009, the concentrations were 0.0886 ± 0.0668 mg/L *vs* 0.0094 ± 0.0210 mg/L). There were no differences in 5-fluorouracil concentrations in renal tissue at each time-point.

CONCLUSION: The use of intraperitoneal 5-fluorouracil with HAES-Steri carrier solution provides a pharmacokinetic advantage for a local-regional killing of residual tumor cells and improve the accumulated penetrability of 5-fluorouracil with decreased systemic toxicity. Further clinical feasibility studies on the use of HAES-steri as carrier solution for intraperitoneal chemotherapy with 5-fluorouracil are warranted.

Key words: Carrier solutions; Intraperitoneal chemotherapy; 5-fluorouracil; Pharmacokinetics; Tissue distribution

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INTRODUCTION

The gastrointestinal cancer tumor is one of the most common clinical malignant tumors[1]. Although the surgical interventions have been advancing and radiotherapy, chemotherapy, biotherapy, immunotherapy and Chinese traditional medicine been developing all the time, the prognosis of patients with gastrointestinal cancer has not been improved obviously and the mortality not been decreased greatly so far, for which the main reason is that the regional recurrence and implantation metastasis can not be treated effectively. The recurrence and metastasis of gastrointestinal cancer often occurs in the resection place, peritoneal membrane surface and liver by turns[2,3]. Intraperitoneal chemotherapy, which aims at this biological behavior and makes the recurrence and metastasis places of gastrointestinal cancer exposed to anti-cancer drug directly for a long time, provides a new adjunctive therapy for intraperitoneal malignant tumors, especially for gastrointestinal cancer now[4,5]. However, two pharmacokinetic problems appear to limit the effectiveness of intraperitoneal therapy: poor tumor penetration and no uniform intraperitoneal distribution by the drug-containing solution[6]. Several factors contribute to the drug distribution but intraperitoneal fluid volume is a dominant factor[7]. Rosenheim *et al* have demonstrated in monkeys that small volumes of fluid do not flow freely in the peritoneum, even with multiple position changes[8]. Volumes large enough to cause moderate abdominal distention result in more uniform intraperitoneal distribution. The ideal carrier solutions for intraperitoneal chemotherapy should expose cancerous surfaces or residual tumor cells within the peritoneal cavity to high levels of cytotoxic agent for as long as possible and make the agents distribute in the abdominal cavity uniformly[9].

Current techniques for intraperitoneal chemotherapy administration most often utilize isotonic micromolecule solutions such as physiological saline, however, the low molecular weight of this solution results in its rapid peritoneal absorption and cannot make the system toxicity from intraperitoneal chemotherapy under satisfactory controll[10]. A successful attempt has been made to prolong retention of intraperitoneal chemotherapy by using icodextrin[11-13], which is an isomolar glucose polymer-based dialysate solution. Another isomolar glucose polymer solution with a potentially long intraperitoneal dwell time is 6% hydroxyethyl starch[14,15].

Slow clearance would benefit the use of cell cycle-specific drugs whose apoptotic effects enhance penetration in solid tumor. Agents undergoing extensive hepatic metabolism such as 5-fluorouracil and doxorubicin possess the greatest regional advantage with intraperitoneal instillation[16-18].

HAES-steri is a neotype hetastarch with middle molecule, which is commonly used for clinical volume expansion therapy. On the basis of its characteristic that it can stay in the blood vessel for a long time and the research about the solutions of the same kind, HAES-steri is promising to be an ideal carrier solution for intraperitoneal chemotherapy. The purpose of these animal experiments was to determine the pharmacokinetics and tissue concentrations of 5-fluorouracil after intraperitoneal perfusion with two isotonic carrier solutions: physiologic saline, a low molecular weight solution, and HAES-steri, a middle molecular weight solution.

MATERIALS AND METHODS

Animals

Male and female Sprague Dawley rats weighing between 200 and 300 g were obtained from a single breeding colony (Laboratory Animal center of Southern Medical University). Animals were individually housed and were allowed free access to food and water. These experiments were conducted after approval by Laboratory Animal Center of Southern Medical University. (the license No. is SY × K 2006-0074).

Surgical procedure

All rats were briefly anesthetized by inhalation of ether (ether, Guanghua Chemistry Co., Ltd. Guangdong, China). Using a 50 mL injection syringe, the cytotoxic agent plus the carrier solution was administered intraperitoneally. The volume of solution administered was 0.1 mL/g body weight. Rats were returned to their cages to recover and were allowed free access to food and water. Rats were anesthetized by inhalation of ether for one minute before the end of the dwell-time. Through a midline thoracolaparotomy the peritoneal fluid was withdrawn completely and quantitated. The blood samples of portal vein and inferior caval vein were taken with a 5 mL injection syringe and 2 mL syringe respectively, and tissue samples were taken from the stomach, colon, liver, kidney and lung.

Experimental design

A total of 60 rats were randomized into two groups according to the carrier solution administered. 5-fluorouracil (Nantong Jinghua Pharmaceutical Co., Ltd., China) was administered in tamed iodine. The dose of drug used in this study was chosen at 100 mg/kg, which exceeds the intraperitoneal dosage used in humans and was meant to be above the analytic detection limit in fluid samples. Based on this dosage, 5-fluorouracil was administered at a concentration of 1000 μg/mL. Two isotonic carrier solutions (0.1 mL/g body weight) were used: physiologic saline (Shenzhou Pharmaceutical Co., Ltd, Guangzhou, China) and 6% hetastarch (Beijing Fresenius Kabi Pharmaceutical Co., Ltd.). Each group was further randomized according to the length of the dwell period of chemotherapy (1, 3, 6, 12, 18 or 24 h). At the end of the procedure the rats were killed. A midline thoracoabdominal incision was made and all peritoneal fluid removed. The volume of peritoneal fluid was recorded and a 0.5 mL

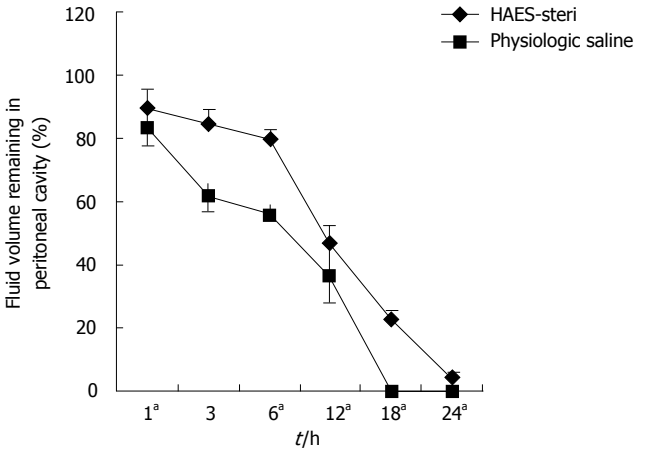

Figure 1 Mean peritoneal fluid volume remaining as a percentage of initial chemotherapy solution volume administered ($^aP < 0.05$ between the two carrier solutions).

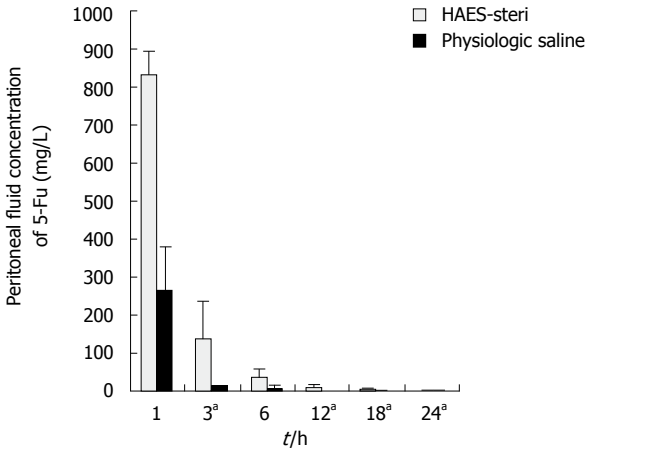

Figure 2 Mean peritoneal fluid concentration of 5-fluorouracil with different carrier solutions ($^aP < 0.05$ between the two carrier solutions).

sample was retained for analysis. Blood and tissue were also sampled. 5-fluorouracil concentrations in plasma, peritoneal fluid, and tissue samples were analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis C

5-fluorouracil levels were determined in plasma, peritoneal fluid and tissue samples, using the HPLC procedures. The HPLC system consisted of a P200 high pressure constant flow pump, an UV-VIS detector set at 265 nm UV, along with an EC2000 color spectrum workstation, an EC2000 Chromatopac data processor. A reversed-phase Diamonsil C_{18} 5 μm silica column 250 mm × 4.6 mm was used, coupled to a guard column of the same chemical consistency (Dikma Technologies, Beijing, China). The mobile phase consisted of an mixture of acetonitrile and ultrapure water (1:19, v/v), run at a flow rate of 1.0 mL/min. Sample injections were 20 μL with 5-bromouracil asinternal standard. All solvents used were HPLC grade (Merck, KGaA).

Sample preparation and analysis

Blood samples were centrifuged and the plasma was separated from the cells. Using a 15-mL polypropylene conical tube, a 500 μL sample of plasma was treated with 100 μL 5-bromouracil as internal standard and 2 mL acetoacetate (Guanghua Chemistry Co., Ltd., Guangdong, China) and mixed thoroughly in a vortex mixer. After centrifugation, the acetoacetate was transferred to another polypropylene tube and evaporated at approximately 40℃ by blowing with a gentle stream of nitrogen. The residue was resuspended in 100 μL mobile phase and filtered through a 0.45 μm syringe filter before HPLC injection. Peritoneal fluid samples were treated as blood sample before HPLC injection.

Tissue samples were processed after drying surface moisture with filter paper. A sample of tissue was accurately weighed with electronic balance and homogenized in ultrapure water with the volume as 3 times as the weight of the sample (v:w = 3 mL/g) with a homogenizing machine. The tissue sample site was consistent for all animals. The homogenate was centrifuged and the supernatant fluid was removed and the following was used as the blood sample.

Statistical analysis

The main parameters of pharmacokinetics and areas under the concentration-time curve were determined using DAS 2.0 (Drug and Statistics Software, Anhui, China). All pharmacokinetic data were compared between groups at each time-point with Mann-Whitney test (two-tailed) using SPSS 10.0. For all statistical procedures, P values < 0.05 were considered significant.

RESULTS

Intraperitoneal volume

Measurements of peritoneal fluid volume at each time-point showed slower clearance from the peritoneal cavity of HAES-steri when compared to physiologic saline (Figure 1). The mean percentage of fluid volume remaining in the peritoneal cavity was significantly higher with HAES-steri at 1, 6, 12, 18 and 24 h (P = 0.047, 0.009, 0.005, 0.005 and 0.005 respectively). No excess peritoneal fluid remained at 18 h with physiologic saline. At 24 h, the percentage of remaining peritoneal fluid volume with HAES-steri was 4.2%.

Peritoneal fluid drug concentration

At each time-point drug concentrations were determined within the peritoneal cavity (Figure 2). The mean peritoneal fluid 5-fluorouracil concentration was significantly greater at 3, 12, 18 and 24 h (P = 0.009, 0.009, 0.005 and 0.005 respectively). There was no significant difference in 5-fluorouracil concentrations of peritoneal fluid between carrier solutions at other time-points.

Plasma drug concentration in portal vein

Plasma 5-fluorouracil concentrations were significantly lower when the drug was administered with HAES-steri at 1 h (P = 0.009) and were significantly higher at 3, 12, 18 and 24 h (P = 0.009, 0.034, 0.005 and 0.019 respectively) (Figure 3A).

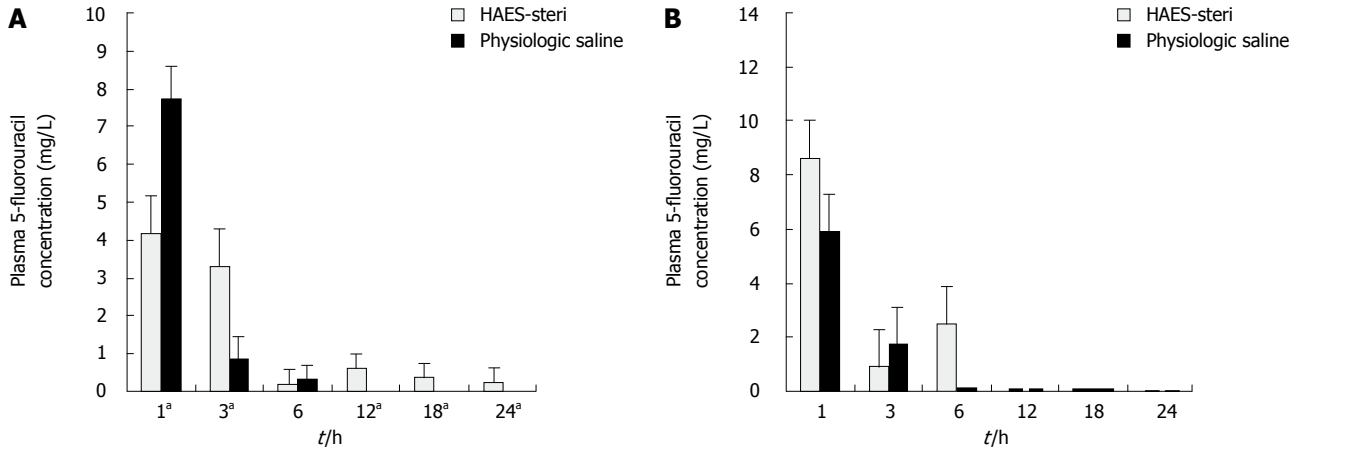

Figure 3 **A**: Mean plasma concentration of 5-fluorouracil in portal vein with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); **B**: Mean plasma concentration of 5-fluorouracil in inferior caval vein with different carrier solutions.

Plasma drug concentration in inferior caval vein

There were no significant differences in plasma 5-fluorouracil concentration in inferior caval vein between different carrier solutions at each time-point (Figure 3B).

Total quantity of drug in peritoneal fluid

The mean total quantity of 5-fluorouracil in the peritoneal fluid decreased with time for both hetastarch and peritoneal dialysis solution, but was significantly greater with hetastarch at 12 h (P = 0.008), 18 h (P = 0.009) and 24 h (P = 0.009) (Figure 4A). No measurable drug or excess peritoneal fluid was present at 18 h when physiologic saline was used. When HAES-steri was used, the mean volume of peritoneal fluid remaining at 24 h was 4.2% ± 1.7% (± SD) of the initial peritoneal fluid volume. The mean total quantity of 5-fluorouracil in this fluid was extremely low (0.7458 ± 0.1954 μg).

Area under the curve ratio of peritoneal fluid to plasma 5-fluorouracil concentration

The area under the concentration over time curve (AUC) ratio with HAES-steri was 1551.095 for peritoneal fluid, 17.49 for plasma in portal vein and 19.466 for plasma in inferior caval vein. The AUC ratio for physiologic saline was 824.054 for peritoneal fluid, 14.516 for plasma in portal vein and 20.275 for plasma in inferior caval vein. The AUC ratio of peritoneal fluid to plasma in portal vein was 88.68 for HAES-steri, and 56.76 for physiologic saline. The AUC ratio of peritoneal fluid to plasma in inferior caval vein was 79.68 for HAES-steri, and 40.64 for physiologic saline. There was an increase of 156% in the AUC ratio of peritoneal fluid to plasma in portal vein and 196% in inferior caval vein with HAES-steri (88.68 *vs* 56.76; 79.68 *vs* 40.64).

Drug concentration in gastric tissue

Mean tissue concentrations of 5-fluorouracil were greater in gastric tissue with HAES-steri. These differences were significant at 18 h (P = 0.016). No significant differences were seen in gastric tissue concentrations of 5-fluorouracil at other time-points (Figure 4B).

Drug concentration in colon tissue

Tissue concentrations of 5-fluorouracil were significantly greater in colon tissue with HAES-steri at 18 h (P = 0.009) (Figure 4C). There were no significant differences in colon tissue concentrations of 5-fluorouracil at other time-points.

Drug concentration in liver tissue

Tissue concentrations of 5-fluorouracil were significantly greater in liver tissue with HAES-steri at 3, 6, 12 and 24 h in liver (P = 0.009, 0.013, 0.034 and 0.013 respectively) (Figure 4D). There were no significant differences in liver tissue concentrations of 5-fluorouracil at other time-points.

Drug concentration in lung tissue

Tissue concentrations of 5-fluorouracil were significantly higher in lung tissue with HAES-steri at 18 h (P = 0.009) (Figure 4E). There were no significant differences in lung tissue concentrations of 5-fluorouracil at other time-points.

Drug concentration in renal tissue

No significant differences were seen in renal tissue concentrations of 5-fluorouracil with the two carrier solutions at each time-point (Figure 4F).

DISCUSSION

Intraperitoneal chemotherapy has shown certain benefits as a treatment for peritoneal surface malignancies and regional recurrence of gastrointestinal cancer after operation so far[19-21]. However, no standard treatment in terms of schedule, dwell-time, drug or carrier solution has been established[9]. It remains an unrealized goal that intraperitoneal chemotherapy makes a great stride in improving the survival and prognosis of patients with gastrointestinal cancer. The lack of ideal carrier solutions is one of the main problems which prevent the progress of intraperitoneal chemotherapy. After radical or palliative surgery for gastrointestinal cancer, tumor recurrence within the peritoneal cavity may be due to residual tumor nodules on the peritoneal surface or to implantation of free cancer cells circulating within peritoneal fluid.

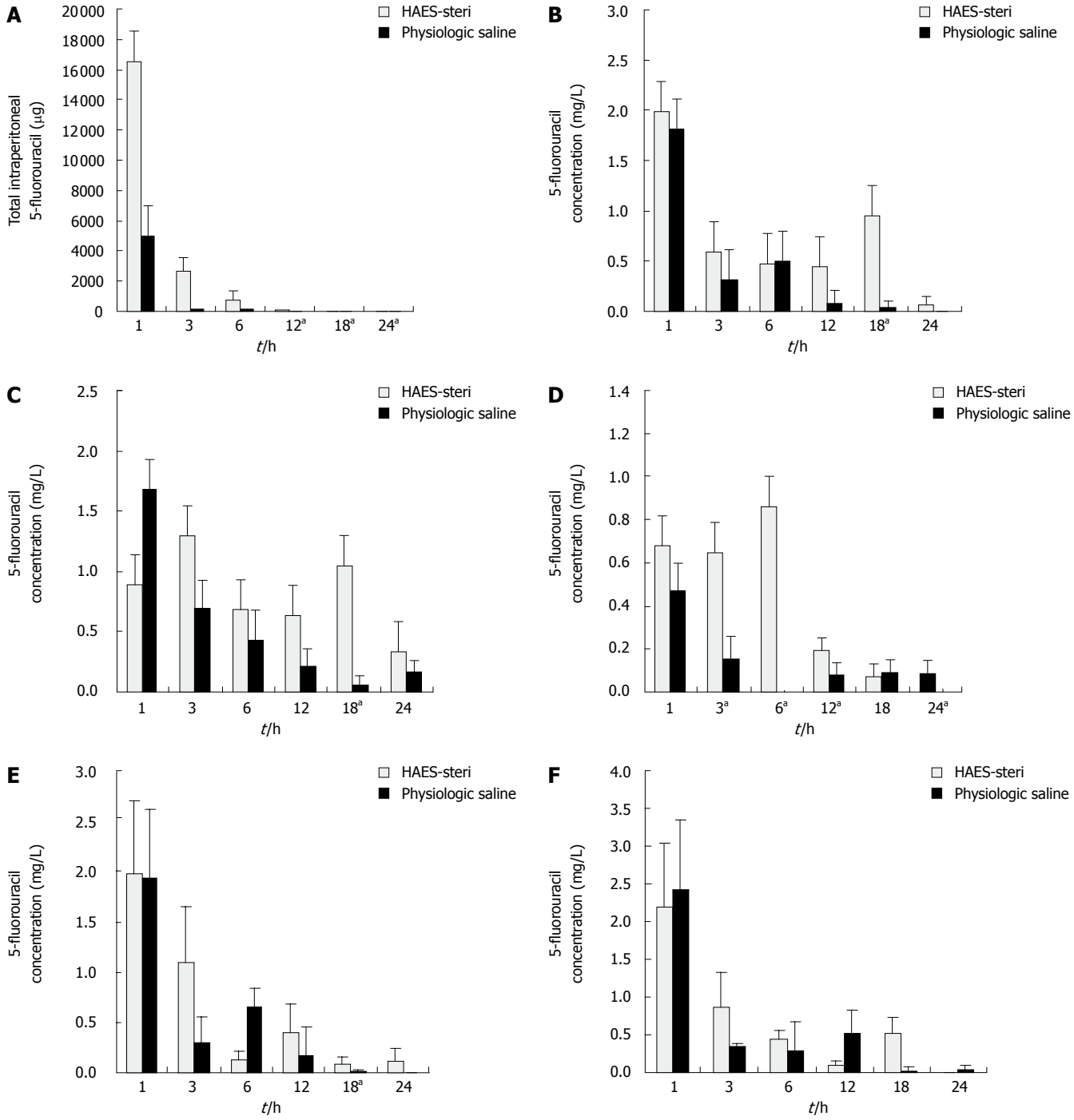

Figure 4 **A**: Mean total quantity of 5-fluorouracil in peritoneal fluid with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); **B**: 5-fluorouracil concentration in gastric tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); **C**: 5-fluorouracil concentration in colon tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); **D**: 5-fluorouracil concentration in liver tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); **E**: 5-fluorouracil concentration in lung tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); **F**: 5-fluorouracil concentration in renal tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions).

Preventing recurrence effectively requires that the tumor nodules have prolonged exposure to the cytotoxic drug. It is necessary for chemotherapy solutions to distribute evenly throughout the entire peritoneal cavity for a prolonged period in order to treat peritoneal and visceral surfaces safely and successfully.

However, isotonic salt solutions, the traditional carrier solutions in use for intraperitoneal chemotherapy, tend to be rapidly absorbed due to their low molecular weight. Pestieau and colleagues have proved that isotonic 0.9% sodium chloride was cleared more rapidly from the peritoneal cavity than high molecular weight solutions and hypertonic sodium chloride, when used as carrier solutions for intraperitoneal chemotherapy with 5-fluorouracil and gemcitabine in an animal model[9]. The inability of isotonic salt or dextrose solutions to maintain a prolonged high intraperitoneal fluid volume limits their effectiveness as carrier solutions for intraperitoneal chemotherapy. The osmolality of the solution may play a role in prolonging the dwell time of intraperitoneal chemotherapy. In a

study by Litterst *et al*, it was shown that slightly hypertonic carrier solutions can prolong the peritoneal retention of chemotherapeutic agents within the peritoneal cavity, probably by inducing a fluid shift inward to the peritoneal cavity[22]. Although the increased accumulation of drugs in tumor cells and enhanced cytotoxicity of cisplatin in hypotonic solution have been confirmed *in vitro* and *in vivo*[23,24], the clinical success with hypotonic solutions as carrier solutions for intraperitoneal chemotherapy has been limited[25,26]. It has been shown that high molecular weight carrier solutions such as Icodextrin and hetastarch have the ability to maintain high intraperitoneal volume for a longer period. Preliminary data receiving intraperitoneal chemotherapy with 7.5% Icodextrin showed that a similar quantity of fluid was drained from the peritoneal cavity as was originally instilled 24 h after administration[27]. However, net fluid flow into the peritoneal cavity may occur when 7.5% icodextrin solutions were used, which could decrease the concentration of drug exposed to cancerous surfaces[6]. A clinical study on the fluid dynamics of 4% icodextrin as carrier solution for intraperitoneal chemotherapy showed that it maintained its instilled volume for up to 48 h, and half the instilled volume remained after 72 and 96 h[28]. Another high molecular weight carrier solution, 6% hetastarch, has also been used in a recent clinical study, which showed reduced clearance of hetastarch from the peritoneal cavity when compared with 1.5% dextrose peritoneal dialysis solution[29].

In the study reported here, the use of HAES-steri, a starch-based carrier solution with middle molecular weight, reduced the clearance of chemotherapy solution from the peritoneal cavity when compared to physiologic saline (0.9% sodium chloride solution). The mean percentage of fluid volume remaining in the peritoneal cavity was significantly higher with HAES-steri at 1, 6, 12, 18 and 24 h. The total quantity of intraperitoneal drug at each time-point was also higher with HAES-steri, especially at 12 h and 18 h. By delaying the clearance of intraperitoneal fluid and thereby maintaining a large distribution, HAES-steri may maximize exposure of cancerous surfaces and optimize intraperitoneal chemotherapy treatments. At the 3, 12 and 18 h time-point, a significantly increased concentration of intraperitoneal 5-fluorouracil was demonstrated. It indicated that HAES-steri could reduce the clearance of 5-fluorouracil from peritoneal cavity and increase the drug concentration exposed to peritoneal surfaces at the same time as this solution maintained high volume in peritoneal cavity for a long time. Accordingly, a larger number of residual tumor cells, minute nodules or free cancer cells in peritoneal cavity can be attacked by high concentrations of anti-cancer drug for over a given time period, which may improve the effectiveness of intraperitoneal chemotherapy for peritoneal regional recurrence of gastrointestinal cancer after surgery.

An important parameter for pharmacokinetic analyses of a drug is the AUC, which represents the total drug exposure integrated over time[10]. The AUC is traditionally the relationship between time and plasma concentration, but can also be applied to concentration of drug in peritoneal fluid for intraperitoneal chemotherapy. Cancer chemotherapy pharmacokinetics assumes a definite relationship of drug response to drug dose. Following intraperitoneal administration of a drug, the AUC reflects the degree of exposure of peritoneal surfaces to chemotherapeutic agent. It is the best estimate of drug delivery and a predictor of response. By comparing the AUC of a drug after intraperitoneal administration in HAES-steri to the AUC after administration in physiologic saline, an estimate of the optimum carrier solution that will prolong contact of peritoneal surfaces and residual tumor cells with chemotherapy solution can be obtained. The ratio of the AUC of 5-fluorouracil in peritoneal fluid to that in plasma of inferior caval vein after intraperitoneal administration reflects exposure of peritoneal surfaces to chemotherapy solution in relation to plasma concentrations of drug, which influences systemic toxicity. The higher AUC ratio of peritoneal fluid to plasma 5-fluorouracil concentration with HAES-steri suggests that better regional exposure of 5-fluorouracil and lower systemic toxicity can be achieved than with physiologic saline. Using intraperitoneal 5-fluorouracil with HAES-steri provides a potential for a favorable antitumor effect on small peritoneal surface tumor deposits or microscopic residual disease.

Another advantage of intraperitoneal chemotherapy is to prevent liver metastasis of gastrointestinal cancer after operation with chemotherapeutics of high concentration in portal vein and liver. In this study, although plasma 5-fluorouracil concentrations were significantly lower when the drug was administered with HAES-steri than with physiologic saline at 1 h, those were significantly higher at 3, 12, 18 and 24 h. Moreover, concentrations of 5-fluorouracil were significantly greater in liver tissue with HAES-steri at 3, 6, 12 and 24 h. This shows that HAES-steri has advantages over physiologic saline as carrier solutions for intraperitoneal chemotherapy to kill free cancer cells in portal vein, consequently, using HAES-steri as carrier solution for intraperitoneal chemotherapy with 5-fluorouracil may improve the effectiveness to prevent liver metastasis of gastrointestinal cancer after surgery.

Tissue concentrations of 5-fluorouracil were significantly higher in gastric and colon tissue at 18-h time-point when HAES-steri was used as carrier solution. This would suggest that HAES-steri increases the accumulated penetrated activity of 5-fluorouracil when used as carrier solution for intraperitoneal chemotherapy, which may benefit the eliminating internal cancer cells in residual tumor nodules. No significant differences were seen in renal tissue concentrations of 5-fluorouracil with the two carrier solutions at each time-point and there were no significant differences in lung tissue concentrations of 5-fluorouraci at time-points except 3-h. These may indicate again that HAES-steri makes the systemic toxicity of intraperitoneal chemotherapy under control when used as carrier solution.

Another advantage of HAES-steri as carrier solution for intraperitoneal chemotherapy is that maintenance of an expanded intraperitoneal space with the use of HAES-steri may additionally ensure separation of loops of bowel to allow direct contact of chemotherapy solution with bowel surfaces prone to adhesion formation and subsequent disease recurrence[30]. The reduction in adhesion formation

has been shown with the use of intraperitoneal 4.5% icodextrin lavage and instillation after laparoscopic gynecologic surgery[18]. The further pathologic study of the impact on the peritoneum and the healing of the operative incision when HAES-steri is used intraperitoneally is needed.

In brief, this study suggests that HAES-steri, by remaining longer in the peritoneal cavity, provides wider intraperitoneal distribution of 5-fluorouracil, and an increased exposure of peritoneal surfaces to anti-cancer drug with lower systemic toxicity than physiologic saline. HAES-steri is a promising carrier solution for intraperitoneal chemotherapy and further clinical feasibility studies on the use of HAES-steri as carrier solution for intraperitoneal chemotherapy with 5-fluorouracil are warranted.

COMMENTS

Background

The principal pharmacokinetic advantage of intraperitoneal chemotherapy over intravenous chemotherapy is the high local drug concentration with low systemic toxicity. The traditional carrier solutions for intraperitoneal chemotherapy fail to optimize this advantage, because these are prone to be cleared from peritoneal cavity rapidly. The ideal carrier solutions should expose cancerous surfaces or residual tumor cells within the peritoneal cavity to high levels of the cytotoxic agent as long as possible.

Research frontiers

It has been shown in published articles that high molecular weight solutions have the ability to maintain high intraperitoneal volume for a longer period, which may offer a number of advantages over low molecular weight solutions. Further study on the pharmacokinetics of intraperitoneal chemotherapy with middle or high molecular weight solutions may solve the problem that there are no ideal carrier solutions for intraperitoneal chemotherapy.

Innovations and breakthroughs

In this article, we studied and compared the pharmacokinetics and tissue distribution of intraperitoneal 5-fluorouracil with HEAS-steri, a neotype 6% hydroxyethyl starch as carrier solution, and physiologic saline (0.9% sodium chloride solution), a traditional carrier solution, in rats, which may provide a promising carrier solution for intraperitoneal chemotherapy.

Applications

This study on the pharmacokinetics and tissue distribution of intraperitoneal 5-fluorouracil with HEAS-steri and physiologic saline offers experimental data for further clinical study and application of HEAS-steri as carrier solution for intraperitoneal chemotherapy.

Terminology

Intraperitoneal chemotherapy, a treatment in which anticancer drugs are put directly into the abdominal cavity through a thin tube, has obvious pharmacokinetic advantages over intravenous chemotherapy when used in palliative therapy for peritoneal carcinomatosis from gastrointestinal carcinoma and prevention of the postoperative peritoneal recurrences and metastases of gastrointestinal cancer.

Peer review

In the present study the effect HAES-steri, as a promising carrier solution for intraperitoneal chemotherapy, was investigated. The study showed that HAES-steri delayed the clearance of intraperitoneal fluid and increased the concentration of 5-fluorouracil in the peritoneal fluid, tissue (especially liver) and portal vein. The manuscript deals with an interesting topic and conclusive results.

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RAPID COMMUNICATION

Prospective cohort comparison of flavonoid treatment in patients with resected colorectal cancer to prevent recurrence

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Abstract

AIM: To investigate biological prevention with flavonoids the recurrence risk of neoplasia was studied in patients with resected colorectal cancer and after adenoma polypectomy.

METHODS: Eighty-seven patients, 36 patients with resected colon cancer and 51 patients after polypectomy, were divided into 2 groups: one group was treated with a flavonoid mixture (daily standard dose 20 mg apigenin and 20 mg epigallocathechin-gallat, n = 31) and compared with a matched control group (n = 56). Both groups were observed for 3-4 years by surveillance colonoscopy and by questionnaire.

RESULTS: Of 87 patients enrolled in this study, 36 had resected colon cancer and 29 of these patients had surveillance colonoscopy. Among the flavonoid-treated patients with resected colon cancer (n = 14), there was no cancer recurrence and one adenoma developed. In contrast the cancer recurrence rate of the 15 matched untreated controls was 20% (3 of 15) and adenomas evolved in 4 of those patients (27%). The combined recurrence rate for neoplasia was 7% (1 of 14) in the treated patients and 47% (7 of 15) in the controls (P = 0.027).

CONCLUSION: Sustained long-term treatment with a flavonoid mixture could reduce the recurrence rate of colon neoplasia in patients with resected colon cancer.

Key words: Flavonoids; Colorectal cancer; Recurrence risk; Intestinal neoplasia; Colon polyps

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INTRODUCTION

Patients with resected colon cancer are at risk of cancer recurrence which depends mainly on the tumor stage[1]. Within 4-5 years after a curative surgical resection about 40%-50% of patients suffer from a tumor recurrence when their initial tumor stage was Ⅱ or Ⅲ according to the International Union against Cancer (UICC) classification[2-4]. Tumor recurrence can manifest itself as a local recurrence at the site of resection, as metachronous tumor growth somewhere else in the colon or as local or distant metastasis. Recurrence in the colon can take three forms of neoplasia: either as incident carcinoma, as incident adenoma or as a mixture of both.

Patients with colon polyps (adenomas, hyperplastic polyps or serrated polyps) who had a polypectomy are also at risk of recurrence[5] .After an index polypectomy these patients can develop incident adenomas in 40% of cases within 3 years depending on the histology of the polyp. The adenoma recurrence is highest for large and multiple adenomas with dysplastic changes of the adenoma structure[5].

There is much controversy about what can be done to reduce the risk or recurrence of neoplasia in tumor and polyp patients. Secondary prevention is urgently needed in these patients; however, it is not yet clear what measures are most effective. Epidemiological studies indicate that dietary interventions with ballast augmented food can be successful for primary prevention of colorectal carcinomas[6]. On the other hand diets supplemented with bran[7] and fruits and vegetables[8] do not suppress the

evolution of colorectal adenomas after polypectomy. Other dietary components such as folic acid, calcium, vitamin D and selenium either have shown only marginal beneficial effects or no effects for prevention[9-12]. Antioxidative vitamins could not prevent gastrointestinal cancer[13]. Beside dietary factors (bioprevention) chemically defined intervention with aspirin[14-17] and nonsteroidal anti-inflammatory drugs (NSAIDs) seem to be effective for primary and secondary prevention of colon neoplasia[18,19]. However, their unwanted side effects and complications (ulcerations, bleedings and thromboembolic events) prevent their general use for risk reduction[20].

Various modes of bioprevention with dietary components have been tested mostly in epidemiological studies[21-23] and in studies with cell culture work and other *in vitro* tests[24]. Few clinical intervention studies were reported. Candidates for use in clinical studies include secondary plant products such as flavonoids, indols, isothiocyanates, glucosinolates, allyes, resveratrol, curcumin, saponins and terpenes. Some of these plant products can be applied as nutritional supplements as tablets, thereby facilitating long-term use without side effects or problems of compliance. Tea flavonoids from green tea and camomile contain flavons, flavonols and flavanols. They have been shown to display various anticarcinogenic, antiproliferative, antimutagenic and antioxidative properties *in vivo* and *in vitro*[24]. Certain species of dietary flavonoids were able to reduce the risk of colorectal cancer[25], even in a dose dependent manner[26]. This is not true for colon neoplasia in general[27,28]. Recently, a clinical study[29] suggested, that the flavonol quercetin taken together with curcumin suppressed the growth of adenomatous polyps in patients with hereditary colon polyposis syndrome (familiar adenomatous polyposis, FAP patients).

We decided to study prospectively the effects of a sustained treatment with a tea-based flavonoid mixture on the evolution of neoplastic alteration in a cohort of high risk patients with resected colon cancer as well as patients after polypectomy. In this proof of principle study we found that a flavonoid intervention can reduce the recurrence rate of neoplasia in patients with sporadic colorectal neoplasia, in comparison with an untreated matched control group.

MATERIALS AND METHODS

Study subjects

Between January 2000 and December 2003 a total of 160 patients with colorectal neoplasia (index patients) were recruited and their data were collected from the clinical charts of the Community Hospital Groß-Gerau, Germany (Department of Internal Medicine and General Surgery) and included into the tumor registry for this clinical study. All patients with the diagnosis of colorectal cancer and colon polyps confirmed by pathology reports were eligible for this study if they completed the clinical questionnaire and had surgical tumor resection or polypectomy. By the end of 2003, recruitment was terminated and until December 2005, 87 patients were followed up by surveillance colonoscopy. Patients who were still in the study at this time were considered to be censored cases for overall survival. All data were extracted from the local tumor registry of the clinic and further pseudonymized for evaluation. In this prospective observational cohort study the investigators had no role in the clinical management; all treatment decisions (except the assignment of the flavonoid nutritional supplementation) and the schedule of surveillance colonoscopies were left at the discretion of the treating physician. Surveillance data were collected prospectively. All 160 patients were treated according to the clinical guidelines for follow-up investigations for colorectal cancer and colon adenomas published by the German Association for Digestive and Metabolic Diseases[30]. This study was approved by the Ethics Committee of the Technical University of Dresden, Germany. The patients provided information using a self-administered questionnaire and in this way written informed consent was obtained authorizing use of their data for this study.

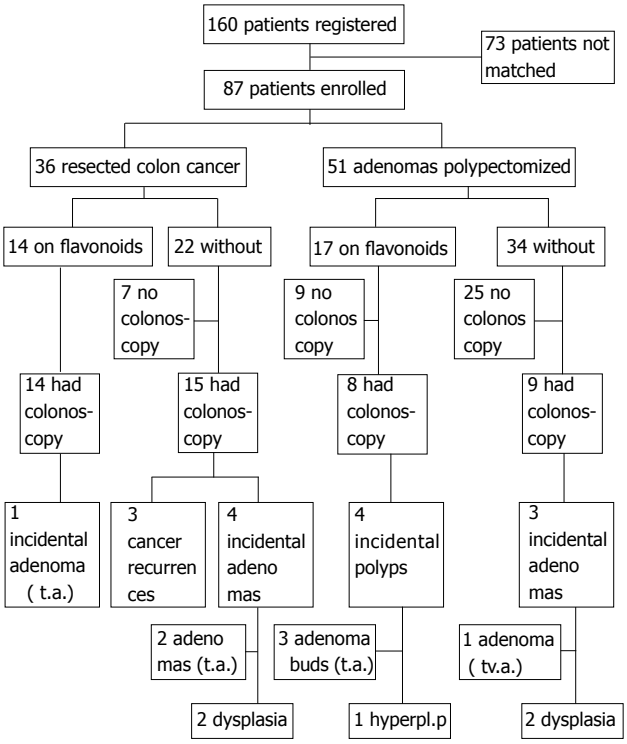

Figure 1 Trial profile and outcomes. t.a.: Tubular adenoma; tv.a.: Tubulovillous adenoma; hyperpl.p: Hyperplastic polyp, buds diminutive polyp (< 5 mm).

Study protocol

Figure 1 explains the trial profile, the outcome and the patient flow of this controlled study. One hundred and sixty patients were registered and of these 87 patients were enrolled to test the efficacy of flavonoids. During the four years from January 2000 to December 2003 we recruited 31 of the 160 patients to agree to take the flavonoid supplement for tumor prevention. Following assignment to treatment these 31 patients were matched to 56 controls. Matching by gender, age (10 years intervals) and type of neoplasia (resected carcinoma *vs* polypectomized adenomas) was performed by using the data of the 129 untreated patients. The remaining 73 patients of the

Table 1 Demographic and outcome data of patients treated with flavonoids and controls

	Treated (n = 31)	Controls (n = 56)	P value
Males/females	17/14	31/25	> 0.9
Age (yr) median (IQR)	74 (68-80)	77 (69-82)	0.35
BMI (kg/m²) median (IQR)	26.1 (24.4-28.2) (n = 28)	27.5 (25.0-30.3) (n = 45)	0.32
Resected colon cancer/polypectomy	14/17	22/34	0.65
Surveillance colonoscopy/no	22/9	24/32	0.014[1]
Surveillance time by colonoscopy			
Years: Median (IQR)	3.5 (3-4.75) (n = 22)	3.0 (2-3) (n = 24)	0.019[1]
Surveillance time by questionnaire			
Years: Median (IQR)	3.6 (3.1-4.7)	2.9 (2.5-3.4)	0.004[1]
Cancer recurrence/no	0/20	3/18	0.23
Polyp recurrence/no	5/15	7/14	0.73
Neoplasia recurrence/no	5/15	10/11	0.20
Smoker/non-smoker	2/27	6/48	0.71
Alcohol/no	24/5	33/20	0.08
Black tea/no	16/15	27/26	> 0.9
Green tea/no	13/16	21/27	> 0.9
Fruit intake < 3/≥ 3 × weekly	8/20	17/35	0.80
Vegetable intake < 3/≥ 3 × weekly	15/13	29/22	0.82
Aspirin use/no	11/20	18/37	0.82
NSAID use/no	2/29	3/52	> 0.9
Colon cancer in family/no	1/30	6/49	0.41
Adenomas in family/no	2/29	1/54	0.29

IQR: Interquartile range (25%-75%); BMI: Body mass index; n: Number of patients; [1]Significantly different at $P < 0.05$.

total of 160, who did not fulfil the matching criteria, were not followed further in this study. The flavonoid-treated patients took a daily dose of 2 tablets of the flavonoid mixture[24] containing 10 mg apigenin and 10 mg epigallocatechin-gallate per tablet. This nutritional supplement (tea bioflavonoids) was produced according to the principles of Good Manufacturing Practice by Köhler-Pharma, Alsbach-Hähnlein, Germany. The content of active ingredients in each batch of the product was tested by chemical analysis (HPLC technique). Flavonoids were taken for 2-5 years; the treatment compliance was evaluated by questionnaire.

Outcomes were evaluated according to the per protocol principle: data of patients using flavonoids (n = 31) were analyzed regardless of how long they had been treated. The primary endpoints of this study were the incident neoplasia (cancer and/or adenomas) observed by surveillance colonoscopy.

The self-administered questionnaire provided information on relevant clinical variables which might influence the clinical outcome. These included life style variables, body mass index (BMI), a dietary food frequency questionnaire, information on medical treatment, cancer and adenoma histories of relatives and tea consumption (Table 1). Data on colonoscopy findings were taken from the standardized clinical endoscopy protocols and transferred to the registry. Histological findings of neoplasia provided by the clinical pathologist were rated according to the guidelines as mentioned above[30]. Tumor stage was assessed from the surgical protocols and rated according to the UICC classification[4].

Statistical analysis

The data of the total cohort of 160 patients were subdivided into the two basic sub-cohorts: patients only observed (n = 73) and patients surveyed for secondary prevention (n = 87). The latter group was divided into a treatment group (n = 31) and a control group (n = 56) as described in the Study Protocol. The patient characteristics of the two surveillance groups, the per protocol group of the treated patients (n = 31) and their controls (n = 56) were compared on baseline as well as for their outcome variables by using descriptive and confirmatory statistical methods. Categorical variables were analyzed using the chi-square test or the 2-sided Fisher Exact Test in the case of small frequencies. Continuous variables (age, BMI) were analyzed using the non-parametric Wilcoxon-Mann-Whitney U-Test. They are described by their median and the interquartile range (IQR). The IQR is defined as the range between the 25th and the 75th percentile of the empirical distribution of the data.

Differences of recurrence were expressed in percentages as absolute differences. The relative risk ratio (RRR) and the number needed to treat (NNT) were computed. Because of the observational nature of this study no adjustments for multiplicity were applied and $P < 0.05$ was considered statistically significant.

RESULTS

The prognostically relevant clinical variables of the treated patients were compared with those of the matched patients (Table 1). During the study period one patient in the treatment group and two patients in the control group died of causes not related to tumor recurrence. The patients in the treated group had significantly higher numbers of follow-up colonoscopies than patients in the control group (Table 1).The time under surveillance both by colonoscopy and by questionnaire was significantly longer for the treatment group (Table 1). The ratio of cancer to polyp patients was not significantly different (45% *vs* 39%) among treatment and control group.

Table 2 Comparison of clinical variables in patients with resected colon cancer on surveillance colonoscopy treated with flavonoids *vs* controls

	Flavonoid treatment (n = 14, %)	Controls (n = 15, %)	P value
Males/females	7/7	7/8	> 0.9
Age (yr) median (IQR)	75.0 (77-82)	81.0 (77-86)	0.12
BMI (kg/m²) median (IQR)	26.2 (24.6-28.0) (n = 13)	25.9 (24.5-27.5) (n = 10)	0.57
Smoker/non-smoker	0/13 (0)	1/12 (8)	> 0.9
Alcohol habitual/no	13/0 (100)	7/5 (58)	0.015[1]
Black tea/no	5/9 (36)	8/5 (61)	0.26
Green tea/no	5/8 (36)	5/6 (45)	> 0.9
Fruit intake < 3/≥ 3 d a week)	2/11 (15)	2/10 (17)	> 0.9
Vegetable intake < 3/≥ 3 d a week)	6/7 (46)	8/4 (67)	0.43
Aspirin/no	4/10 (28)	7/7	0.44
NSAID/no	0/14 (0)	1/13 (7)	> 0.9
Colon *vs* rectum cancer	13/1 (93)	9/6 (60)	0.080
Low *vs* high tumor stage (Ⅰ and Ⅱ/Ⅲ)	9/5 (64)	9/6 (60)	> 0.9
Surveillance time by colonoscopy Years: median (IQR)	4.0 (3.25-5)	3.0 (2-3)	0.022[1]

IQR: Interquartile range (25%-75%). [1]Significantly different at $P < 0.05$.

Table 3 Recurrence rates of colon neoplasia in patients with resected colon cancer treated with flavonoids compared to controls

	Treated (% of total, n = 14)	Controls (% of total, n = 15)	Absolute difference (%)	RRR	NNT	P value
Cancer recurrence/no	0/14 (0)	3/12 (20)	20		5	0.125
Adenoma recurrence/no	1/13 (7)	4/11 (27)	20	3.9	5	0.101
Neoplasia recurrence/no	1/13 (7)	7/8 (47)	40	6.7	2.5	0.027[1]

RRR: Relative risk ratio; NNT: Number needed to treat. [1]Significantly different at $P < 0.05$.

Recurrence rates of cancer were 0 in 20 in the treated group *vs* 3 in 21 in the control group (P = 0.23) Polyp recurrence rates were 5 in 20 in the treatment group *vs* 7 in 21 in the control group (P = 0.73). The combined rate of recurrence for neoplasia was 5 in 20 in treated *vs* 10 in 21 in the controls (P = 0.20). These differences are not statistically significant, but there is a trend for more favourable outcomes in the flavonoid exposed patients. Note that both groups were not adjusted according to surveillance colonoscopy and according to neoplasia type. The sample size of this proof of principle study is small. Also, it can be seen in Figure 1, that the incident polyps in the control group were high grade adenomas (4 adenomas with dysplasia, one tubulovillous adenoma); there were only 2 tubular adenomas. Among the treated patients there were 3 diminutive tubular adenomas (polyp buds), one hyperplastic polyp and one tubular adenoma (with 10 mm diameter). This shows that there were more advanced adenomas present in the control group than in the treatment group.

Fruit consumption of less than 3 d a week was considered as low intake and was found in 29% (8 in 28) of the treatment group as compared to 33% (17 in 52) of the control group (P = 0.80). Habitual vegetable intake of less than 3 d a week was reported by 54% (15 in 28) of patients in the treatment group *vs* 57% (29 in 51) in the control group. Habitual drinking of green and black tea was not significantly different among both groups; about 44% drank green tea and 51% black tea. About 10% of the patients in both groups smoked and about 30% of them took aspirin regularly. NSAIDs were taken long term by 5%-6% of the patients in both groups. Habitual alcohol use was reported by 83% in the treatment group as compared to 62% in the control group (P = 0.08). Gender, age and BMI were approximately evenly distributed among the two groups.

Most patients in the flavonoid group (20 in 31) took the nutritional supplement for more than 12 mo, 8 patients took it less than 3 mo, 2 up to 6 mo and one patient up to 12 mo. Three in 27 (11%) reported slight discomfort and discontinued the flavonoid treatment within 3 mo. The majority of 65% (17 in 26) took the flavonoids continuously on a daily basis.

As the data in Table 1 suggested that there is a possible treatment effect of the use of flavonoids we analyzed our data in the well adjusted group of patients with curative colon cancer resection. There were 14 patients with resected colon cancer in the treatment group compared to 15 control patients (Table 2); all had surveillance colonoscopies. None of the treated patients had cancer recurrence *vs* 20% (3 in 15) of the controls. Among the controls two patients had metastatic colorectal cancer and one had local cancer recurrence at the surgical anastomosis. The time to relapse was 2-3 years after surgery in patients with cancer recurrence. Adenomas developed in 7% (1 in 14) of the treated patients and in 27% (4 in 15) of the controls including two adenomas with dysplasia (Table 3). There was a statistically significant difference (P = 0.02) between the two groups when the combined endpoint of neoplasia recurrence (incident cancer and incident adenomas) was evaluated. The potentially confounding patient characteristics of both groups did not differ

significantly except for habitual alcohol consumption, which was significantly more prevalent in the treated patients than in controls. For neoplasia recurrence the prognostically most important factor is the previous tumor stage, which was not significantly different between the two groups.

DISCUSSION

Recurrence risk is the main concern of patients with previous resected colorectal cancer[1-4]. On follow-up about 40% of surgically curable colorectal cancers with stage Ⅱ and stage Ⅲ (according to the UICC staging system) will suffer recurrent cancers within 3-4 years. The best outcomes were reported for stage I and stage Ⅱ tumors (around 90% survival without recurrences). The prognosis of stage Ⅲ cancer (with cancerous regional lymph nodes) is less favourable, but can be improved by adjuvant chemotherapy. Treated cases and controls in our study did not differ regarding the initial tumor stage at surgery; about 40% in both groups were stage Ⅲ tumors, only 2 of them (controls) had adjuvant chemotherapy because the surgeon felt confident that most of these patients would not be suitable for adjuvant chemotherapy. The tumor recurrence in the controls was not observed in the patients on chemotherapy, but there were too few patients to judge whether this could influence outcomes. We found the expected recurrence rate in the controls (Table 3), but no incident cancers and only one incident adenoma in the flavonoid exposed patients. Eighty-seven of the 160 patients from the registry were enrolled because we detected only 56 controls that could be properly adjusted to the 31 treated patients. The matching ratio of about one to two (31 treated *vs* 56 controls) seems to be appropriate. In this real world study 76% of the treated and 43% of the controls had surveillance colonoscopies; among the resected patients 80% had surveillance by colonoscopy but only 33% of the polypectomized patients. This fact might influence the reliability of the conclusions regarding the adenoma recurrence.

Our controlled clinical trial was a prospective and observational cohort study performed with the aim of finding out whether long-term flavonoid exposure of patients from a tumor registry alters the outcome compared to untreated control patients. This proof of principle study suggests that flavonoids can be used to reduce the recurrence rate in patients with resected colorectal cancers. Flavonoids are good candidates for primary and secondary prevention of colorectal cancer, since numerous *in vitro* studies and animal work report on their beneficial activities in terms of suppression of cancer proliferation, antioxidative and antiangiogenetic properties[24]. Epidemiological investigations[22,25,26], *in vivo* and *in vitro* experiments[31-35] and one clinical intervention study[29] support this concept. Other authors could not find protective effects of flavonoids on colorectal cancer incidence[21,27,36]. Flavonoids derived from tea plants can be used as a mean of bioprevention and have been manufactured and marketed as nutritional supplements[24]. Other methods of prevention are not effective (e.g. vitamins except folic acid), show only marginal efficacy (e.g. calcium, selenium) or cannot be used in general because of their unwanted side effects and complications (aspirin, NSAIDs)[20].

We tested the efficacy of flavonoid supplementation in a high risk population (resected colorectal cancer) to examine its effect in a relatively small number of patients, which were carefully adjusted for various clinical variables with prognostic relevance. However, there are prognostic clinical factors which were not taken into account such as penetration depth into the colonic wall and histological grading. Clinical studies with a larger sample size and a higher statistical power are necessary to show that flavonoid exposure alters the outcome in terms of tumor recurrence. Flavonoids could prevent recurrences of neoplasia by protecting the genome of colonocytes from genotoxic insults such as oxidative damage, free radical attacks and adduct formation[37]. Flavonoids are secondary plant products which could be responsible for some of the healthy effects of fruits and vegetables. It is still unknown which components of vegetables and fruits are effective for tumor prevention; ballast, fibres and secondary plant products play a major role[6,10,38]. Flavonoids, indols, isothiocyanates, curcumin, resveratrol, glucosinolates and other plant products affect carcinogenic, mutagenic and neoplastic mechanisms[24], but could also induce protective enzymes of the intestinal mucosa[39]. Beside the type of chemical and biological prevention lifestyle factors, type and amount of tea consumption, genetic factors, aspirin and NSAID medication could influence the outcome. These variables have to be considered when evaluating the effects of flavonoid intervention. As shown in Tables 1 and 2 these variables were well balanced among cases and controls. However, alcohol use was more prevalent in the treated patients with resected colorectal cancer than in controls. We do not think that differences of habitual alcohol drinking can explain the difference of recurrence since ethanol is thought of as a carcinogenic risk factor and would rather increase the recurrence risk of the flavonoid exposed patients.

Patient compliance with the flavonoid treatment was evaluated using information derived from a questionnaire given to 31 treated patients in the treatment group. 67% of these treated patients took the nutritional supplement longer than 12 mo, only 10% discontinued the intake within the first 3 mo. No side effects or unwanted symptoms were reported.

The habitual vegetable intake of the patients in both treatment and control groups (Tables 1 and 2) was rather low (< 3 d a week) and only about 40%-50% of the patients consumed vegetables ⩾ 3 d a week, which still is not sufficient for tumor prevention. About 16%-30% of the patients (cases and controls, Tables 1 and 2) reported low fruit content in their diet (< 3 d a week). Thus, no significant differences of the dietary habits were observed among treated and untreated patients. The self-administered questionnaire which was used to assess dietary habits provided only a crude estimate and was not validated; it is however a simple and practical tool that was well accepted and understood by the patients.

Flavonoids are part of human nutrition and are contained in vegetables and fruits, especially in apples, onions, berries, citrus fruits and teas but also in chocolate. Tea consumption of the patients was moderate and was

reported in most cases only as occasional tea drinking.

More patients with resected colorectal carcinoma of the control group (7 of 14, 50%) took aspirin compared to the cases (4 of 14, 28%) but this difference was not statistically significant (Table 2).

Surveillance by colonoscopy was performed in more cases (65%) than in controls (38%) and the time interval covered by colonoscopy was longer in treated patients than controls (Table 1). Thus the treated patients had a better chance for detection of neoplasia which would be a bias against a treatment effect. If the controls had more surveillance intensity, their recurrence rate would have been even higher.

In patients with prior adenomas that were removed by polypectomy and had surveillance colonoscopies, those treated with flavonoid treatment had a polyp recurrence rate similar to that of controls (about 50%). However, flavonoid treatment was associated with low risk incident adenomas while the control group included polyp recurrence of two adenomas with dysplasia (Figure 1). These differences were not statistically significant but could indicate that flavonoids could also suppress adenoma development and evolution. Cruz-Correa *et al* have recently reported that a combined treatment with quercetin (a flavonol) and curcumin (from curry) inhibited proliferation of adenomas in patients with familiar adenomatous polyposis coli[29]. These point to the possibility that flavonoids taken as long-term treatment could suppress neoplasia recurrence in high risk patients.

In conclusion, this pilot study which was controlled, prospective and observational, suggests that long-term flavonoid treatment could reduce the recurrence rate of colon neoplasia in high risk patients particularly in those with resected colorectal cancer. Therefore flavonoid supplementation should be investigated by further clinical studies to prove the efficacy and validity of this concept.

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COMMENTS

Background

Recurrence of cancer after a curative surgical resection in patients with colorectal cancer is a common problem that occurs in about 20%-40% depending on the previous tumor stage. It is essential for these patients to find ways to prevent this disaster.

Research frontiers

Prevention of recurrence can be achieved by adherence to a diet containing lots of fruits and vegetables or for higher tumor stages by cytostatic chemotherapy (adjuvant chemotherapy). Chemotherapy is very demanding and prone to unpleasant side effects. Dietary measures are difficult to implement and could give rise to bloating, gas and pain of the abdomen.

Innovations and breakthroughs

Other authors and articles seem to suggest that flavonoids could prevent colorectal cancers by healthy dietary habits, e.g. intake of foods with a high content of flavonoids. All these studies rely on epidemiological data and these are not always consistent and sometimes controversial. Our study uses an interventional approach with a nutritional supplement (as tablets) and this has not been done previously. Our data suggest that all patients at risk of recurrence of colorectal cancer should be treated with flavonoid supplements.

Peer review

It is a well-designed paper. The authors showed that sustained long-term treatment with a flavonoid mixture could reduce the recurrence rate of colon neoplasia in patients with resected colon cancer. This is an interesting article.

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RAPID COMMUNICATION

A red wine polyphenolic extract reduces the activation phenotype of cultured human liver myofibroblasts

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Abstract

AIM: To test the effect of a standardized red wine polyphenolic extract (RWPE) on the phenotype of human liver myofibroblasts in culture.

METHODS: Human myofibroblasts grown from liver explants were used in this study. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Signaling events were analyzed by western blot with phospho-specific antibodies. Matrix-metalloproteinase activity was measured with gel zymography.

RESULTS: We found that cell proliferation was dose-dependently decreased by up to 90% by RWPE while cell viability was not affected. Exposure to RWPE also greatly decreased the phosphorylation of ERK1/ERK2 and Akt in response to stimulation by the mitogenic factor platelet-derived growth factor BB (PDGF-BB). Finally, RWPE affected extracellular matrix remodeling by decreasing the secretion by myofibroblasts of matrix-metalloproteinase-2 and of tissue inhibitor of matrix-metalloproteinases-1.

CONCLUSION: Altogether, RWPE decreases the activation state of liver myofibroblasts. The identification of the active compounds in RWPE could offer new therapeutic strategies against liver fibrosis.

Key words: Liver fibrosis; Myofibroblasts; Hepatic stellate cells; Wine; Phosphorylation; Proliferation

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INTRODUCTION

Liver fibrosis is a serious health problem worldwide. It is a complication of most chronic liver diseases whether due to excessive alcohol consumption, chronic viral hepatitis B or C, non alcoholic steatohepatitis, hemochromatosis or others. The pathophysiology of liver fibrosis has been extensively studied (recently reviewed in[1,2]). Whatever the initial insult, the abundant extracellular matrix (ECM) characteristic of liver fibrosis is synthesized by myofibroblastic cells. Myofibroblasts are mostly absent from the normal liver but at least two types of resident liver cells can be differentiated into myofibroblasts during liver disease: hepatic stellate cells, and portal fibroblasts[3]. Myofibroblastic differentiation is characterized by a high rate of cell proliferation and of ECM synthesis and by cytoskeletal changes, notably expression of alpha smooth muscle actin (ASMA) that confers contractile properties to the cells[4]. In addition, degradation of the normal liver ECM results from an increased secretion of the enzyme matrix metalloproteinase-2 (MMP-2) by myofibroblasts, while the proteolytic degradation of the abnormal ECM is inhibited due to a high level synthesis of a MMP inhibitor, tissue inhibitor of MMP-1 (TIMP-1)[1,2]. In the recent years, a series of natural products were shown to be of potential benefit against liver fibrosis[5,6]. For instance, we and others found that a polyphenolic component of red wine, trans-resveratrol, was able to strongly deactivate liver fibrogenic cells[7,8], while a related molecule, trans-piceid, was uneffective[8]. However, red wine contains many other polyphenolic substances, and red wine polyphenolic extracts (RWPE) showed many interesting biological effects in other settings, notably in the prevention of experimental atherosclerosis[9]. One of the mechanisms postulated in this context is related to the inhibitory effect of RWPE on the proliferation of vascular smooth muscle cells[10]. Since vascular smooth muscle cells share many characteristics with myofibroblasts, we tested the hypothesis that RWPE would affect the phenotypic characteristics of human liver myofibroblasts, especially those related to their pro-fibrogenic activity.

MATERIALS AND METHODS

Materials

Preparation and characterization of the polyphenolic extract (RWPE) from a red French wine (Corbières, A. O. C.) was as described previously[11,12]. One liter of red wine produced 2.9 g of extract, which contained 471 mg/g total phenolic compounds expressed as gallic acid. Phenolic levels in the extract were obtained according to HPLC analysis procedure. In particular, the extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, dimers (B1: 6.9 mg/g; B2: 8.0 mg/g; B3: 20.7 mg/g; and B4: 0.7 mg/g), anthocyanins (malvidin-3-glucoside: 11.7 mg/g; peonidin-3-glucoside: 0.66 mg/g; and cyanidin-3-glucoside: 0.06 mg/g), and phenolic acids (gallic acid: 5.0 mg/g; caffeic acid 2.5 mg/g; and caftaric acid: 12.5 mg/g). Stock solutions (1 mg/mL) were prepared in distilled water containing 1% ethanol and further diluted in culture medium. All dilutions were adjusted so as to contain 0.1% ethanol, and medium with 0.1% ethanol was used as a control.

Fetal calf serum (FCS) was from Dutscher (Brumath, France) and human serum from Etablissement Français du Sang (Bordeaux, France). Epidermal growth factor (EGF) was from Peprotech (Tebu, Le Perray en Yvelines, France) and recombinant platelet-derived growth factor BB (PDGF-BB) was from Eurobio (les Ulis, France). Phospho Thr308 AKT-1 and AKT-1 antibodies, phospho-ERK1/ERK2 and total ERK antibodies were from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). ASMA and vimentin antibodies were from Dako (Trappes, France). The TIMP antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Beta-actin antibody was from Sigma (Saint Quentin Fallavier, France). IRDye 680 and IRDye 800 conjugated secondary antibodies; Odyssey Blocker and Odyssey infrared imaging system were from LI-COR Biosciences (ScienceTec, Les Ulis, France).

Cell isolation and culture

Human hepatic myofibroblasts were obtained from explants of non-tumor liver resected during partial hepatectomy and characterized as described previously[13,14]. Specifically, the procedure, based on the selective growth advantage of myofibroblasts in the culture conditions used, allowed for 100% pure myofibroblasts population, as shown by positive staining for ASMA and vimentin, and negative staining for CD68 (a Kupffer cell marker), von Willebrand factor (an endothelial cell marker) or cytokeratin (an epithelial cell marker). This procedure is in accordance with INSERM ethical regulation imposed by French legislation. Myofibroblasts were used between the 3rd and the 6th passage, and were grown in DMEM containing 5% FCS, 5% pooled human serum and 5 ng/mL EGF. EGF was removed from the medium at least 3 d before experiments.

Cell proliferation assay

Cells were seeded at a density of 10^4/well in 24-well plates. On the following day, the medium was replaced by DMEM with 5% FCS and RWPE dilutions to be tested. After three or seven days, the medium was removed and the cells incubated with 1 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] for 2 h at 37℃[15]. The crystals were then dissolved with DMSO and the optical density was recorded at 540 nm. Results were expressed as proliferation index = (B-A)/(C-A) where A is the optical density recorded at d 0, B is the optical density with the test compound and C is the optical density obtained in the control wells.

Measure of cell DNA content

Cells were grown to confluence in 24-well plates, serum-starved for 24 h, then incubated with RWPE. After 24 h, the cells were lysed with NaH_2PO_4/Na_2HPO_4 50 mmol/L, pH 7.4, NaCl 2 mol/L, EDTA 2 mmol/L, and DNA content was measured as described[16].

Western blot

ERK and Akt phosphorylation was measured essentially as described previously[17]. Briefly, cells were grown to confluency and serum-starved for three days. They were then pre-incubated with RWPE in serum-free Waymouth medium for 1 h, and then exposed to 20 ng/mL PDGF-BB for 10 min. At the end of the incubation, cell lysates were prepared in the presence of proteases and phosphatases inhibitors as described[18]. Proteins were measured with a Bio-Rad assay. Equivalent amounts of proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by two color Western blotting with antibodies to total-ERK and phospho-ERK, or phospho-Akt-1 and β-actin. Blots were blocked in Odyssey Blocker and incubated simultaneously with both primary antibodies, followed by both IR-labeled secondary antibodies. Signals were detected and quantified using the Odyssey infrared imaging system.

The expression of ASMA and of vimentin was measured in cell extracts from cells exposed for seven days to RWPE using Western blot as described[8].

The concentration of TIMP-1 in conditioned medium was also measured by Western blot. Confluent cells were incubated for two days with RWPE, the medium was collected, centrifuged and aliquots, normalized for DNA content of the cell layers, were analyzed by Western blot.

Zymography for MMP-2 activity

The detection of MMP-2 in conditioned medium was performed by gelatin zymography[19], essentially as previously described[8,20]. Briefly, cells were grown to confluence in 24-well plates, serum-starved for 24 h, then incubated with RWPE. The medium was collected, centrifuged and aliquots, normalized for DNA content of the cell layers, were analyzed on 8% SDS-PAGE gels containing 1 mg/mL gelatin. Following staining with Coomassie blue, MMP activity is detected as a white zone on a blue background.

Immunofluorescence detection of alpha-smooth muscle actin

Cells were seeded at a density of 10^4/well on glass coverslips in 24-well plates. On the following day, the medium was replaced by DMEM with 5% FCS and RWPE

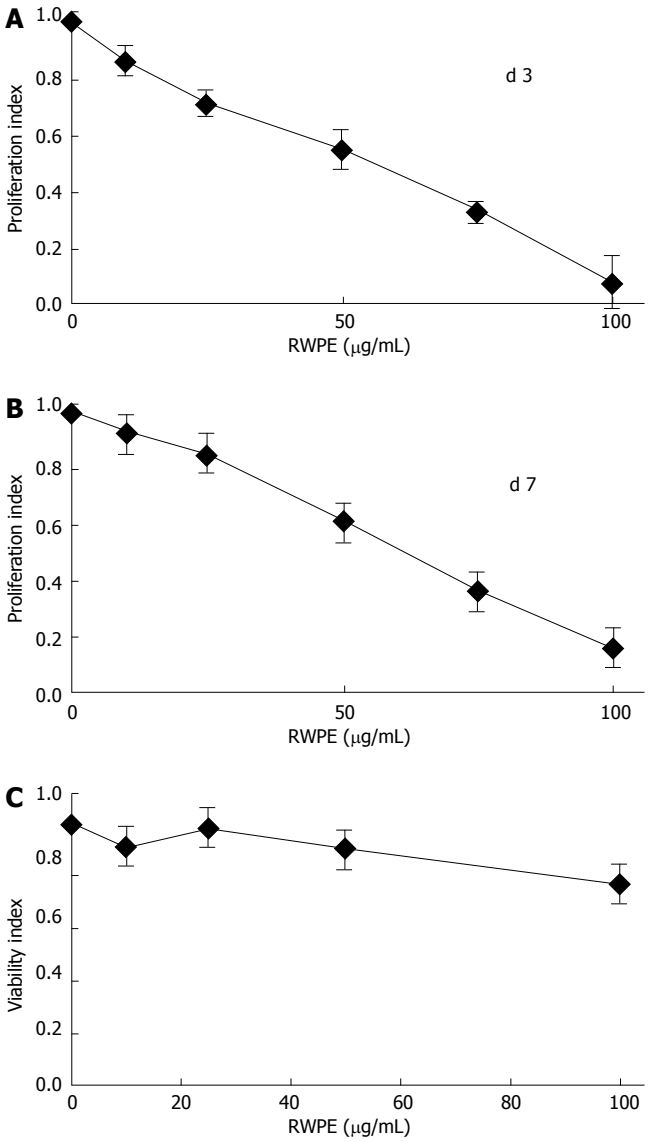

Figure 1 RWPE decreases myofibroblasts proliferation. Myofibroblasts were grown for either three (**A**) or seven days (**B**) in the presence of the indicated concentrations of RWPE. Results are the mean ± SD of 4 independent experiments conducted in quadruplicate. The effect of RWPE was highly significant using ANOVA ($P < 0.0001$). In control experiments, myofibroblasts were exposed to RWPE for 24 h and the DNA content of the cell layer was measured (**C**). Results are expressed as the percentage of the values in treated cells as compared to cells treated with the solvent alone and are the mean ± SD of 3 independent experiments conducted in quadruplicate. There were no significant differences between conditions.

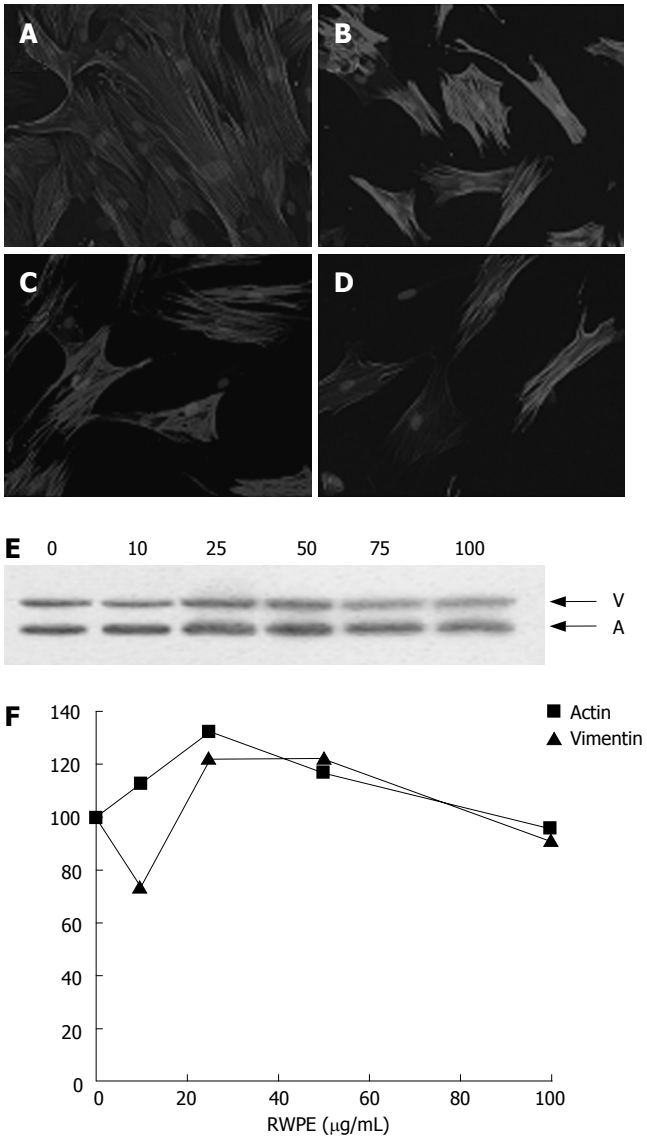

Figure 2 Effect of the RWPE on expression of alpha-smooth muscle actin. **A**: Myofibroblasts were incubated for seven days in the absence of RWPE (A) or with 50 (**B**), 75 (**C**), or 100 (**D**) μg/mL RWPE. Aliquots of cell extracts grown in the same conditions were analyzed by Western blot (**E**) simultaneously for ASMA (A) and vimentin (V). **F**: The signals were quantified as described in Materials and Methods. The graph shows the mean of 2 separate experiments.

dilutions to be tested. After three days, cells were fixed with methanol at -20℃, then incubated sequentially with an anti-ASMA monoclonal antibody and a Texas Red-conjugated secondary antibody. The slides were examined with a Zeiss Axioplan fluorescence microscope.

RESULTS

Effect of the RWPE on cell proliferation

The RWPE dose-dependently inhibited the proliferation of myofibroblasts down to 7.8% ± 4.5% of control at d 3 and 15.8 ± 8.0 at d 7 (Figure 1A and B). The concentration that reduced growth by 50% was 50 μg/mL. A toxic effect of RWPE on cells could be ruled out because no morphological signs of toxicity nor cell detachment were observed (Figure 2); furthermore, when confluent cells were exposed to a dose range of RWPE, there was no decrease in DNA content of the cell layers even at the highest concentrations (Figure 1C).

Effect of the RWPE on expression of alpha-smooth muscle actin

Expression of the cytoskeletal protein ASMA is hallmark of activated liver fibrogenic cells. We found that long term (up to seven days) exposure of liver myofibroblasts to RWPE did not affect ASMA expression. This was shown both by immunofluorescence and by Western blot (Figure 2). In addition, the expression of another cytoskeletal protein, vimentin, which expression is independent of fibrogenic cell activation, was also unaffected by RWPE treatment (Figure 2C).

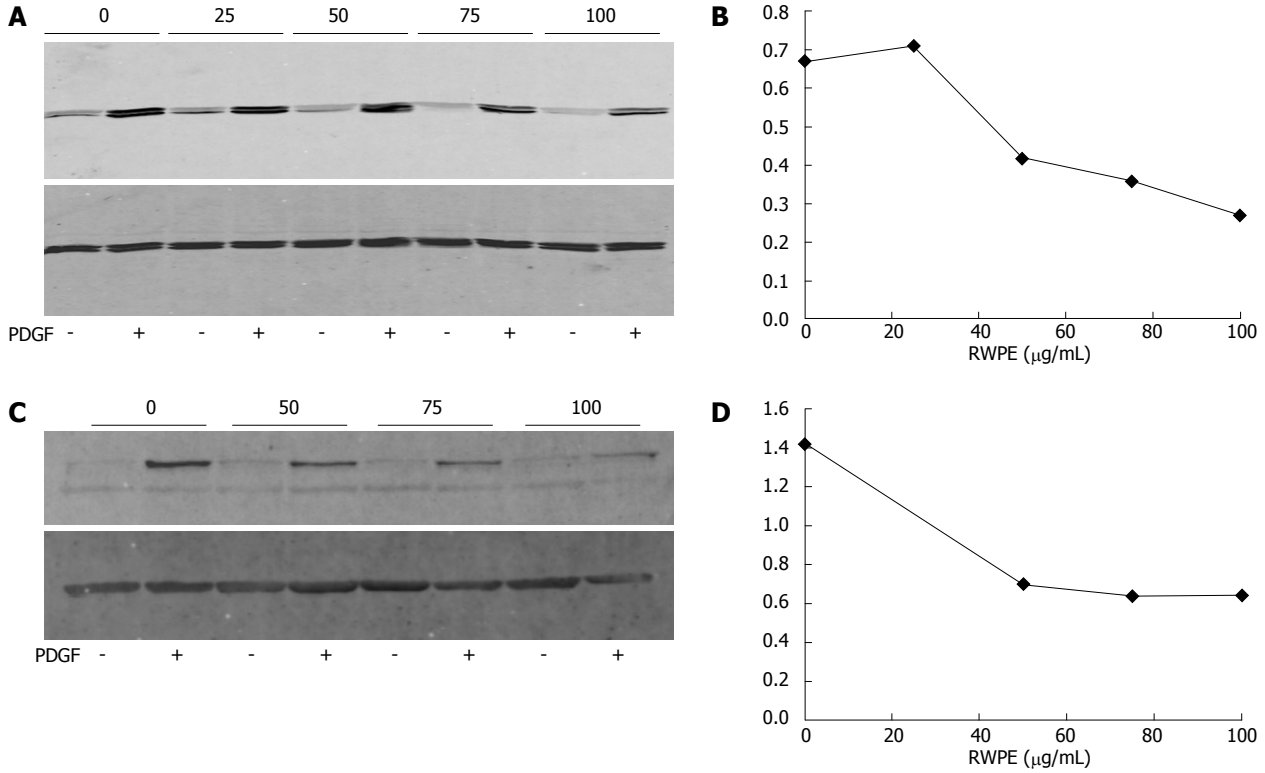

Figure 3 Effect of the RWPE on the phosphorylation of MAPK and Akt. **A**: Myofibroblasts were pre-incubated for 1 h with the indicated concentrations of RWPE (in μg/mL) or solvent, then exposed for 10 min to 20 ng/mL PDGF-BB or buffer. Identical amounts of cell extracts were analyzed by Western blot with antibodies to phospho-ERK1/ERK2 (top panel) and to total ERKs (bottom panel). The picture is representative of 3 experiments; **B**: Quantitative analysis of the experiment shown in (**A**). The activation index refers to the ratio between the levels of phospho-ERK to those of total ERK; **C**: Same as in A except that the blot was labelled with an anti-phospho-Akt antibody (top panel) and an antibody to β-actin (bottom panel); **D**: Quantitative analysis of the experiment shown in (**C**). The activation index refers to the ratio between the levels of phospho-Akt to those of β-actin.

Effect of the RWPE on the phosphorylation of MAPK and Akt

In order to delineate the mitogenic pathways affected by RWPE, myofibroblasts were briefly exposed to PDGF-BB. PDGF-BB is major mediator of liver fibrogenic cell activation, as it stimulates notably their proliferation[13,21] and migration[22,23], and is abundant in serum. We then examined the effect of RWPE on signalization pathways elicited by PDGF-BB. As expected, treatment with PDGF-BB induced a major increase in the phosphorylation of ERK1/ERK2 and of Akt. Exposure to RWPE greatly decreased the effect of PDGF-BB on the phosphorylation of both ERK1/ERK2 and Akt (Figure 3).

Effect of the RWPE on MMP-2 and TIMP-1 expression

A high level expression of the matrix remodeling enzyme MMP-2[24], and of the inhibitor of extracellular matrix degradation, TIMP-1[25], is characteristic of activated liver fibrogenic cells. Gelatin zymography showed a gelatinolytic band migrating at an apparent molecular weight of 72 kDa characteristic of MMP-2. The RWPE strikingly and dose-dependently decreased the secretion of MMP-2 (Figure 4A). It also greatly decreased TIMP-1 secretion, as assessed by Western blot (Figure 4B).

DISCUSSION

We show here that a standardized RWPE has striking effects on the phenotype of human liver myofibroblasts. This is shown by a decreased proliferation rate together with decreased secretion of MMP-2 and of TIMP-1. These effects are not the consequence of a direct toxicity of the extract on cells as shown by morphological examination and DNA content measurement. We investigated further the mechanism of the decreased proliferation rate and found that RWPE treatment largely abolished the phosphorylation of ERK1/ERK2 and of Akt induced by the mitogenic factor PDGF-BB. Previously, Iijima *et al* showed that a RWPE decreased Akt but not ERK activation in vascular smooth muscle cells stimulated with PDGF[26]. The differences may be due to the fact that different RWPEs were used, or to a true cell specificity. For instance, despite the fact that myofibroblasts and smooth muscle cells are related cells, we found that they had a differential response to trans-resveratrol[8]. Some of the effects of the RWPE are reminiscent of those of trans-resveratrol, raising the possibility that RWPE effects may be due to this compound. However, resveratrol is present only at low concentration in wine (reviewed in[27]) and is unlikely to explain the effects of RWPE. In addition, whereas it does indeed decrease myofibroblasts proliferation, MMP-2 secretion[8] and Akt activation[28], it does not affect ERK activation in response to PDGF[28]. Furthermore, contrary to RWPE, it does decrease ASMA expression[8].

The RWPE effects observed in the present study are potentially of benefit against liver fibrosis, if they held true in the *in vivo* situation. This seems obvious for the reduced cell proliferation that will decrease the number of ECM-

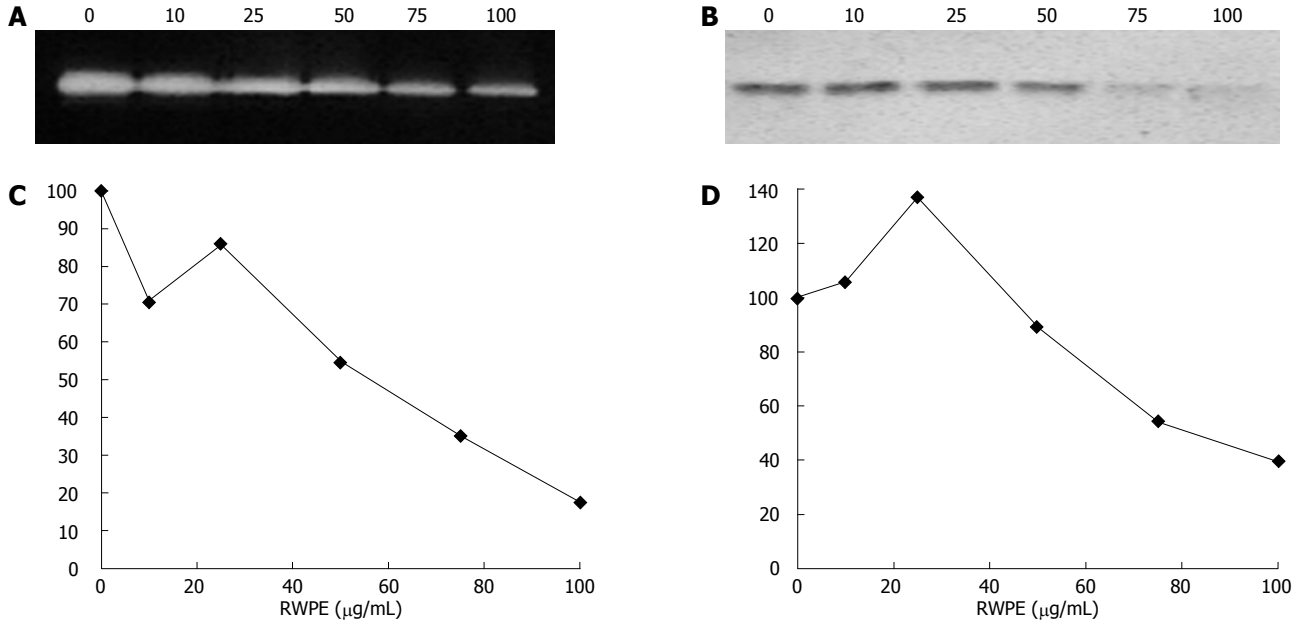

Figure 4 Effect of the RWPE on MMP-2 and TIMP-1 expression. **A**: Myofibroblasts were incubated for 24 h with the indicated concentrations of RWPE (in μg/mL). Aliquots of conditioned medium normalized for the DNA content of the cell monolayers were analyzed on gelatin-containing gels (**A**). The white bands on the dark background indicate gelatinolytic activity. Other aliquots were analyzed by Western blot with an antibody against TIMP-1 (**B**). Another experiment gave similar results; **C**: Quantitative analysis of the experiments. MMP-2: Mean of duplicate samples; TIMP-1: Mean of 2 independent experiments.

producing cells. Notably, the drastic effect on TIMP-1 secretion may have a major implication since TIMP-1 overexpression is considered one of the main determinants of liver fibrosis through the inhibition of ECM-degrading enzymes activity[29,30].

Thus, although excessive wine consumption is one of the major causes of chronic liver diseases, wine itself may unexpectedly contain potent anti-fibrotic compounds. The identification of the active compounds in RWPE could offer new therapeutic strategies against liver fibrosis.

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We thank PL Teissedre for providing the standardized RWPE.

COMMENTS

Background

Liver fibrosis is a worldwide problem, as it complicates all chronic liver diseases. There is no established treatment for liver fibrosis.

Research frontiers

A series of natural products have shown beneficial effects on liver fibrosis in cell culture and animal models. In addition, red wine polyphenols were shown to reduce the proliferation of vascular smooth muscle cells, a cell type closely related to liver fibrogenic cells.

Innovations and breakthroughs

We found that a standardized red wine polyphenolic extract greatly decreased the proliferation of human liver fibrogenic cells. It also reduced their synthesis of matrix-metalloproteinase-2 and of the tissue inhibitor of matrix metalloproteinase-1, thus suggesting that it could affect the cell ability to remodel the extracellular matrix.

Applications

The identification of the active compound(s) in the extract could lead to *in vivo* testing of its anti-fibrotic activity in liver disease.

Terminology

Liver fibrosis: a common complication of most chronic liver diseases, where an excess of extracellular matrix components are deposited in the liver.

Peer review

This is a very nice communication and provides mechanistic data, is well-written, and ends with a thorough discussion. The results of this study are novel and potentially lay the ground work for the understanding of the effect of wine/wine extracts on human hepatic fibrosis.

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RAPID COMMUNICATION

Effects of estradiol and progesterone on the proinflammatory cytokine production by mononuclear cells from patients with chronic hepatitis C

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Abstract

AIM: To investigate the effects of estradiol (E2) and progesterone on the unstimulated and oxidative stress-stimulated production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, and macrophage chemotactic protein (MCP)-1 by peripheral blood mononuclear cells (PBMCs) from patients with chronic hepatitis C and healthy controls.

METHODS: The PBMCs were separated from age-matched 72 males and 71 females with and without chronic hepatitis C, who were divided into two groups based on a mean menopausal age of 50 years. Oxidative stress was induced by hydrogen peroxide in the cells incubated in serum-free media. Cytokines in the culture supernatant were measured by an enzyme-linked immunosorbent assay.

RESULTS: The highest levels of the spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by the unstimulated PBMCs were in the older male patients with chronic hepatitis C and the lowest levels were in the pre-menopausal female healthy controls. E2 inhibited the cytokine production by the unstimulated PBMCs from the older male and post-menopausal female patients, which was further stimulated by progesterone. The exposure to hydrogen peroxide in the PBMCs from the younger male and pre-menopausal female healthy subjects induced the production of cytokines. The change rates of the hydrogen peroxide-stimulated cytokine production were suppressed by E2 and enhanced by progesterone.

CONCLUSION: These findings suggest that E2 may play a favorable role in the course of persistent liver injury by preventing the accumulation of monocytes-macrophages and by inhibiting proinflammatory cytokine production, whereas progesterone may counteract the favorable E2 effects.

Key words: Estradiol; Progesterone; Mononuclear cell; Proinflammatory cytokine; Chemokine

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INTRODUCTION

In inflammatory and oxidative liver injury, the accumulation of leukocytes and macrophages including Kupffer cells on the sites of injury and inflammation in the liver is thought to be mediated by such cytokines as chemokines, including interleukin (IL)-8 and macrophage chemotactic protein (MCP)-1. These monocytes and macrophages are in turn

able to release proinflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1β, thus leading to the occurrence of persistent liver injury. It has been reported that chronic hepatitis C virus (HCV) infection tends to progress more rapidly in men than in women[1,2], and that the decline in estradiol (E2) production with menopause is associated with a spontaneous increase in TNF-α and IL-1β[3]. It should be noted that large increases in the amount of reactive oxygen species (ROS) lead to disturbance of prooxidant-antioxidant balance, or oxidative stress. Our previous studies have shown that hepatocytes possessed estrogen receptor (ER), and that in cultured hepatocytes in a state of oxidative stress, E2 inhibited the activation of the nuclear factor κB (NF-κB), a key transcription factor that induces multiple genes in response to inflammation and oxidative stress[4], through ER[5,6]. These findings suggest that E2 could enhance the anti-inflammatory activity in the injured liver by decreasing the proinflammatory cytokine production, and it might, therefore, play a cytoprotective role through ER in the liver[7,8]. However, regarding E2 and another female sex steroid, progesterone, there is little information about the direct effects of these sex hormones on the production of chemokines and proinflammatory cytokines by monocytes and macrophages in chronic HCV infection, although *in vitro* experiments in which monocytes were incubated with sex hormones have revealed conflicting results regarding monocyte cytokine production[9]. Therefore, this study investigated the effects of E2 and progesterone on the production of TNF-α, IL-1β, IL-8, and MCP-1 by the heparinized peripheral blood mononuclear cells (PBMCs), after stimulation with the ROS, hydrogen peroxide[10]. These effects were then compared between the blood samples from the age-matched male and female patients with chronic hepatitis C and the healthy controls, which were divided into two groups based on a mean menopausal age of 50 years.

MATERIALS AND METHODS

Patients

This study was conducted at Tokushima University Hospital and the subjects consisted of age-matched males (n = 72) and females (n = 71) including 71 patients with chronic hepatitis C and 72 healthy controls who were divided into younger, or pre-menopausal (< 50 years of age), and older, or post-menopausal (≥ 50 years of age) groups. The chronic hepatitis patients met the following criteria: a persistently elevated serum alanine aminotransferase (ALT, normal serum levels of ALT range between 5 and 40 U/L) level for at least six months; HCV-RNA seropositivity; seronegativity for Hepatitis B virus (HBV) surface antigen; exclusion of all other potential cause of chronic liver disease such as autoimmune hepatitis, primary biliary cirrhosis, drug-induced hepatitis, or metabolic liver disease; and no history of alcohol abuse, defined as an alcohol intake of more than 20 g per day over the previous year. In addition, any individuals taking oral contraceptives or corticosteroids, who had previously undergone an operation on the ovaries were also excluded from the study.

All females in the younger groups with and without chronic HCV infection had a normal ovarian cycle, and the mean ovarian cycle length was 28 d with a range of 26 to 32 d. Ethical approval was obtained from the Tokushima University Hospital ethics committee, and informed consent was obtained from all patients taking part in the study.

The heparinized peripheral blood samples were obtained in the morning after an overnight fast. In the pre-menopausal females, the blood samples were taken during the luteal phase 5 to 8 d before the onset of menses. The human PBMC fractions were separated using density-gradient centrifugation with a lymphocyte separation medium (Organon Teknika, Durham, NC, USA). After three washes with phosphate-buffered saline and buffered RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% L-glutamine (RPMI culture medium), the mononuclear cells were suspended at a concentration of 1×10^6/mL in RPMI culture medium with 10% heat-inactivated fetal bovine serum. The cells were cultured at 37℃ in a 5% CO_2 atmosphere and 100% humidity for 24 h and then treated with and without 17β-E2 (Sigma, St Louis, MO, USA) or progesterone (Wako, Osaka, Japan) for another 6 h in the presence and absence of the estrogen receptor antagonist, ICI-182, 780 (ICI: Tocris Cookson, Ballwin, MO, USA), or the PR antagonist RU486 (RU: Wako). In another experiment, the culture medium was changed with serum-free RPMI, and oxidative stress was induced by hydrogen peroxide (10^{-7}-10^{-5} mol/L). The cells were then incubated with either E2 or progesterone for up to 6 h in the presence and absence of ICI or RU. The steroid sex hormones and receptor antagonists were initially prepared as an ethanol stock solution (10^{-2} mol/L) and then were diluted with the culture medium in order to obtain an appropriate working solution concentration.

Cytokine and female sex hormone assays

The cytokines of TNF-α, IL-1β, IL-8 and MCP-1 and the female sex hormones of E2 and progesterone secreted into the culture supernatant were measured by an enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D Systems, Minneapolis, MN, USA) and by a radioimmunoassay using commercial kits (CIS Diagnostic, Tokyo, Japan), respectively, according to the manufacturer's instructions.

Statistical analysis

The data were presented as the mean ± SD, unless otherwise indicated. The means were compared between the two groups using the Wilcoxon's signed-rank test and the Mann-Whitney U test with Bonferroni correction. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Characteristics at the time of isolation of PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

As shown in Table 1, the background factors of 4 patient groups with chronic hepatitis C, such as the serum levels of

Table 1 Characteristics at the time of isolation of PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

	Patients with chronic hepatitis C				Healthy controls			
	Males (yr)		Females (yr)		Males (yr)		Females (yr)	
	< 50	⩾ 50	< 50	⩾ 50	< 50	⩾ 50	< 50	⩾ 50
n	18	18	17	18	18	18	18	18
Age (yr)	41 ± 7	69 ± 6	41 ± 7	68 ± 6	40 ± 6	68 ± 6	40 ± 6	69 ± 6
HCV-RNA (log copies/mL)	6.1 ± 0.8	6.5 ± 1.4	5.9 ± 0.8	6.4 ± 1.2	ND	ND	ND	ND
ALT (U/L)	118 ± 68	112 ± 52	105 ± 49	101 ± 55	27 ± 8	26 ± 8	24 ± 7	24 ± 8
Platelet count (10^3/mm^3)	208 ± 58	189 ± 52	206 ± 54	185 ± 55	230 ± 60	219 ± 53	225 ± 57	214 ± 58

The PBMCs were isolated from age-matched males (*n* = 72) and females (*n* = 71) of 71 patients with chronic hepatitis C and 72 healthy controls, who were divided into younger or pre-menopausal (< 50 years of age) and older or post-menopausal (⩾ 50 years of age) groups, based on a mean menopausal age of 50 years. The values are the mean ± SD (*n* = 17 or 18). ND: Not detected.

Table 2 Spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

Subjects with and without chronic HCV infection			TNF-α	IL-1β	IL-8	MCP-1
			(pg/mL supernatant)			
Males	Younger	Patients	354 ± 201[a]	253 ± 142	918 ± 499	403 ± 216
	(< 50 yr)	Controls	265 ± 161	180 ± 105	780 ± 420	308 ± 161
	Older	Patients	385 ± 234[a]	284 ± 160[a]	1087 ± 576[a]	461 ± 242[a]
	(⩾ 50 yr)	Controls	322 ± 200	217 ± 125	899 ± 480	364 ± 187
Females	Pre-menopausal	Patients	288 ± 177	198 ± 111	780 ± 410	334 ± 174
	(< 50 yr)	Controls	210 ± 126	167 ± 94	624 ± 335	275 ± 140
	Post-menopausal	Patients	314 ± 189	264 ± 149[a]	887 ± 473	387 ± 198
	(⩾ 50 yr)	Controls	241 ± 148	182 ± 100	699 ± 372	362 ± 201

The PBMCs were isolated from age-matched males (*n* = 72) and pre- and post-menopausal females (*n* = 71) of 71 patients with chronic hepatitis C and 72 healthy controls. The levels of TNF-α, IL-1β, IL-8, and MCP-1 in the culture supernatant were detected by means of an ELISA. The values are the mean ± SD (*n* = 17 or 18). [a]$P < 0.05$ *vs* the pre-menopausal healthy controls.

ALT and HCV-RNA, and platelet counts (normal ranges between 150 and 350 × 10^3/mm^3), were not significantly different among the younger (< 50 years of age), or pre-menopausal, and the older (⩾ 50 years of age), or post-menopausal groups. There was no significant difference of platelet counts between the patient groups and healthy control groups. Because platelet counts in chronic hepatitis C patients have been reported to be an indicator of the degree of hepatic fibrosis[11], most of the patients in this study seemed to show mild hepatic fibrosis.

Comparison of the spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

PBMCs were isolated from age-matched younger and older males and pre- and post-menopausal females of the patients with chronic hepatitis C and the healthy controls. In the premenopausal females, the blood samples were taken during the luteal phase of the ovarian cycle. The 24-h cultured PBMCs released TNF-α, IL-1β, IL-8, and MCP-1 into the culture medium (Table 2). The levels of E2 and progesterone in the culture supernatant were found to be under 10^{-12} mol/L. Although the net cytokine levels were considerably different among the individuals, the spontaneous production levels of cytokines in the unstimulated PBMCs appeared to show different tendencies between the 8 subgroups, the highest levels were present in the older male patients and the lowest levels were found in the pre-menopausal female controls ($P = 0.039$ for TNF-α, $P = 0.018$ for IL-1β, $P = 0.013$ for IL-8, and $P = 0.011$ for MCP-1). The chronic hepatitis C patients showed higher production levels of TNF-α, IL-1β, IL-8, and MCP-1 as compared with the age-matched healthy controls, and the mean production levels of cytokines in the older groups were higher than those in the younger groups, while the female subjects tended to produce much less cytokines from the unstimulated PBMCs than did the age-matched male subjects (Table 2).

Effects of E2 and progesterone on spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by unstimulated PBMCs from age-matched older male and post-menopausal female patients with chronic hepatitis C

We next investigated the effects of E2 and progesterone on the augmented production of TNF-α, IL-1β, IL-8, and MCP-1 by the unstimulated PBMCs from the age-matched older male and post-menopausal female patients with chronic hepatitis C. When the unstimulated PBMCs were cultured for another 6 h without female sex hormones or receptor antagonists, the mean percentages of each initial value for the cytokine production reached up to 110%-123% (Table 3). Whereas the treatment with E2 and progesterone in the unstimulated PBMCs for 6 h significantly affected the change rates of the cytokine production in a dose dependent manner, the percentages

Table 3 Effects of E2 and progesterone on spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by unstimulated PBMCs from age-matched older male and post-menopausal female patients with chronic hepatitis C

Chronic hepatitis C subjects	TNF-α	IL-1β	IL-8	MCP-1
			(% of initial value)	
Older male patients				
None	123 ± 14	120 ± 16	119 ± 12	114 ± 15
+ E2	82 ± 10[a]	84 ± 10[a]	80 ± 11[a]	78 ± 11a
+ E2 + ICI	118 ± 16	114 ± 18	108 ± 19	99 ± 19
+ Progesterone	167 ± 19[a]	169 ± 20[a]	159 ± 18[a]	169 ± 28[a]
+ Progesterone + RU	129 ± 21	124 ± 21	132 ± 19	122 ± 24
Post-menopausal female patients				
None	118 ± 15	119 ± 14	122 ± 16	110 ± 13
+ E2	83 ± 11[a]	79 ± 9[a]	81 ± 12[a]	74 ± 11[a]
+ E2 + ICI	108 ± 14	122 ± 16	117 ± 19	104 ± 19
+ Progesterone	167 ± 13[a]	170 ± 24[a]	159 ± 20[a]	175 ± 30[a]
+ Progesterone + RU	130 ± 27	125 ± 28	132 ± 30	123 ± 25

The unstimulated PBMCs from the age-matched older male and post-menopausal female patients with chronic hepatitis C were cultured for another 6 h with and without 10^{-8} mol/L E2 (E2) or 10^{-7} mol/L progesterone (progesterone) in the presence and absence of 10^{-6} mol/L ICI (ICI) or 10^{-6} mol/L RU (RU). The levels of TNF-α, IL-1β, IL-8, and MCP-1 in the culture supernatant were detected by means of an ELISA. The results were expressed as the percentages of each initial value for the cytokine production in the absence of the female sex hormones and receptor antagonists. The values are the mean ± SD (n = 18). [a]$P < 0.05$ *vs* 6-h-cultures in the absence of the female sex hormones and receptor antagonists.

of each initial value decreased significantly in the PBMCs treated with E2 at 10^{-8} and 10^{-7} mol/L, and they increased significantly in the PBMCs treated with 10^{-7} mol/L progesterone (Figure 1). There was no significant difference between the change rates of the cytokine production in the male and female patients with chronic hepatitis C.

The inhibitory effects of 10^{-8} mol/L E2 on the unstimulated cytokine production in the male and female patients were blocked by the specific ER antagonist ICI at a dose of 10^{-6} mol/L, while the further enhancement effects of 10^{-7} mol/L progesterone on the unstimulated cytokine production in both genders were blocked by the PR antagonist RU (Table 3). The treatment with ICI or RU alone had no effect on any of the parameters examined herein (data not shown).

Effects of E2 and progesterone on hydrogen peroxide-stimulated production of TNF-α, IL-1β, IL-8, and MCP-1 by PBMCs from age-matched younger male and pre-m enopausal female healthy controls

The exposure to low doses of hydrogen peroxide (10^{-7}-10^{-5} mol/L) in the PBMCs from the age-matched younger male and pre-menopausal female healthy controls, incubated in serum-free RPMI for 6 h, was observed to stimulate the production of TNF-α, IL-1β, IL-8, and MCP-1 in a dose-dependent manner (data not shown). The subsequent studies used a dose of 10^{-5} mol/L of hydrogen peroxide for further stimulation of the incubated PBMCs. The exposure to hydrogen peroxide induced a time-dependent and transient cytokine production, peaking at 1-6 h, over a 6 h period (Figure 2). There was no significant difference between the cytokine levels in the male and female healthy controls. The cytokine levels in the culture supernatant (Figure 2) peaked after 6 h. Subsequent studies used an incubation time of 6 h to measure the levels of TNF-α, IL-1β, IL-8, and MCP-1 after the hydrogen peroxide exposure.

The hydrogen peroxide-stimulated cytokine production was inhibited by 10^{-8} mol/L E2 (Table 4). The inhibitory effect of E2 was blocked by 10^{-6} mol/L ICI (Table 4). In contrast to E2, progesterone treatment for 6 h resulted in the further cytokine production in the oxidative stress-stimulated PBMCs. The stimulatory effect of progesterone (10^{-7} mol/L) was blocked by 10^{-6} mol/L RU (Table 4). No parameters examined in the PBMCs were found to be significantly different between the male and female subjects.

DISCUSSION

In the present study, the highest levels of the spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by the unstimulated PBMCs were found to be in the older male patients with chronic hepatitis C, and the lowest levels were in the pre-menopausal female healthy subjects, although the cytokine levels were considerably different among the individuals. The male subjects tended to produce cytokines from the unstimulated PBMCs to a much greater degree than did the age-matched female subjects. The augmented cytokine production by the PBMCs from the older male and post-menopausal female patients with chronic hepatitis C was inhibited by supplementation with E2, and was further stimulated by supplementation with progesterone through their receptors, when the unstimulated cells were cultured for an additional 6 h. The exposure to low doses of hydrogen peroxide in the PBMCs from younger male and pre-menopausal female healthy subjects incubated in the serum-free media for 6 h was observed to induce cytokine production. The change rates of the hydrogen peroxide-stimulated production of TNF-α, IL-1β, IL-8, and MCP-1 in the PBMCs were suppressed by E2, and were enhanced by progesterone through their receptors. The specificity of the E2-mediated anti-inflammatory induction through the ER and the progesterone-mediated proinflammatory induction through the PR was shown

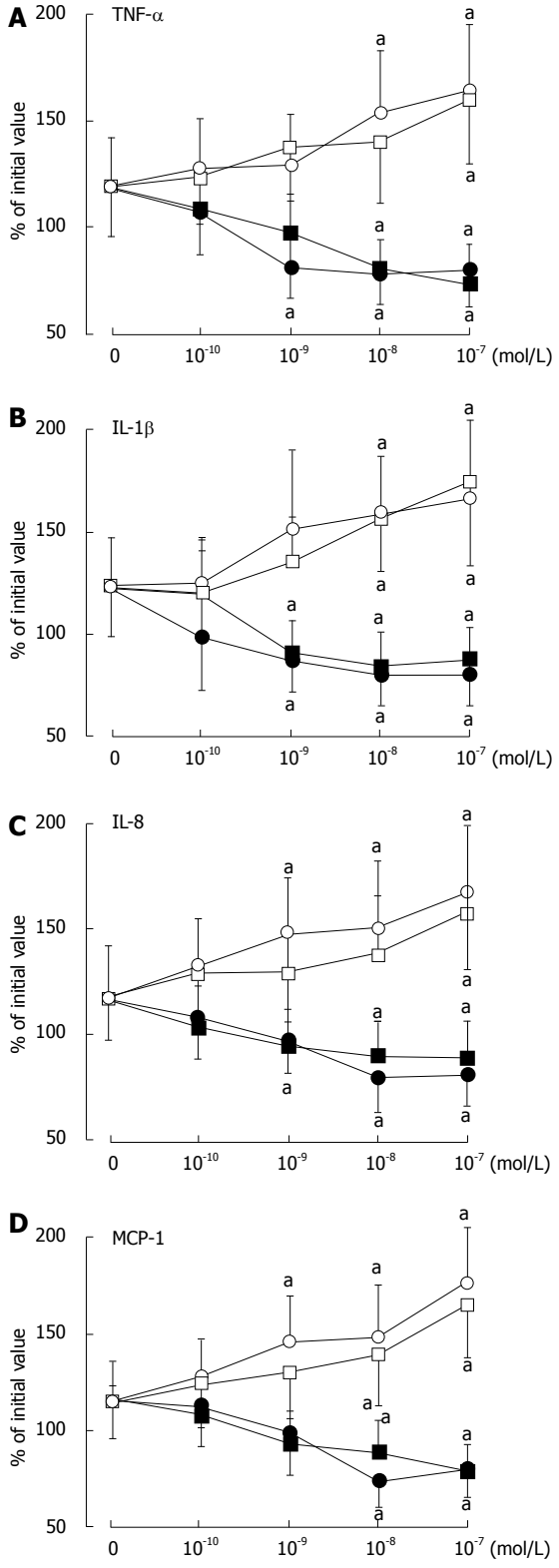

Figure 1 Effects of E2 and progesterone on spontaneous production of TNF-α (**A**), IL-1β (**B**), IL-8 (**C**), and MCP-1 (**D**) by unstimulated PBMCs from age-matched older male and post-menopausal female patients with chronic hepatitis C. The unstimulated PBMCs from the age-matched older male (black square) and post-menopausal female (black circle) patients with chronic hepatitis C were cultured for another 6 h with and without E2 (10^{-10}-10^{-7} mol/L) (solid) or progesterone (10^{-10}-10^{-7} mol/L) (open). The spontaneous production levels of TNF-α (**A**), IL-1β (**B**), IL-8 (**C**), and MCP-1 (**D**) in the culture supernatant were detected by means of an ELISA. The results were expressed as the percentages of each initial value for the cytokine production in the absence of the female sex hormones. The values are the mean ± SD (n = 18). $^{a}P < 0.05$ in comparison to the 6-h-cultures in the absence of the female sex hormones.

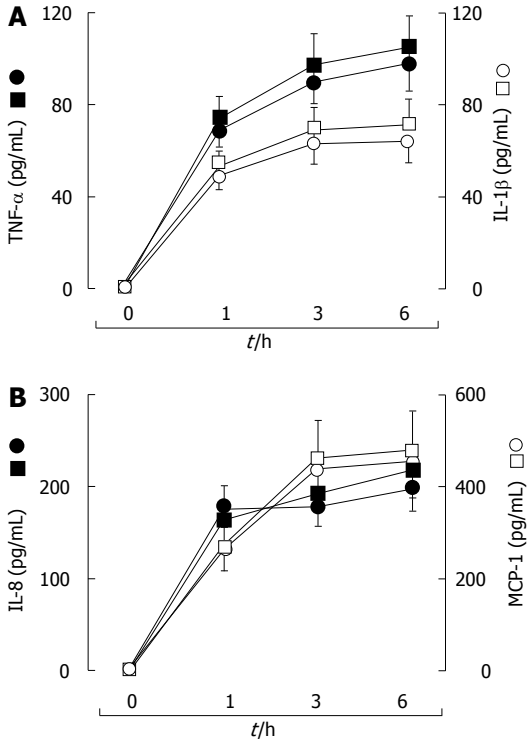

Figure 2 Stimulation of TNF-α, IL-1β (**A**), IL-8, and MCP-1 (**B**) production after exposure to hydrogen peroxide by unstimulated PBMCs from age-matched younger male and pre-menopausal female healthy controls. The unstimulated PBMCs from the age-matched younger male (black square) and pre-menopausal female (black circle) healthy controls were incubated for up to 6 h in serum-free RPMI in the presence of 10^{-5} mol/L hydrogen peroxide. The levels of TNF-α (**A** solid), IL-1β (**A** open), IL-8 (**B** solid), and MCP-1 (**B** open) in the culture supernatant were detected by means of an ELISA. The values are the mean ± SD (n = 10).

by ICI and RU, respectively, in both the unstimulated and oxidative stress-stimulated PBMCs. The inhibitory effect of E2 at a dose of 10^{-8} mol/L on the unstimulated and stimulated cytokine production was blocked by ICI in both gender subjects. Treatment with the progesterone receptor antagonist RU led to a blockage of further cytokine production induced with 10^{-7} mol/L progesterone by the unstimulated and stimulated PBMCs from both genders. No parameters examined in the PBMCs were found to be significantly different between the male and female subjects.

There is a large body of evidence indicating that the decline in the ovarian function with menopause is associated with spontaneous increases in TNF-α, IL-1β, and IL-6[3]. E2, at physiological concentrations (10^{-11}-10^{-8} mol/L), has been reported to inhibit the spontaneous secretion of these proinflammatory cytokines in whole blood cultures[12] or PBMCs[13]. The unstimulated production of TNF-α and IL-1β in PBMCs has been reported to be higher in patients with chronic hepatitis C than in healthy subjects[14]. These findings were consistent with the present data. The *in vivo* treatment with E2 transdermally in postmenopausal women has been reported to decrease the spontaneous IL-6 production by PBMCs after 12 mo of the therapy[13]. One preliminary study also showed the hydrogen peroxide-induced TNF-α

Table 4 Effects of E2 and progesterone on hydrogen peroxide-stimulated production of TNF-α, IL-1β, IL-8, and MCP-1 by PBMCs from age-matched younger male and pre-menopausal female healthy controls

Healthy subjects	TNF-α	IL-1β	IL-8	MCP-1
		(pg/mL supernatant)		
Younger male controls				
Oxidative stress	102 ± 17	66 ± 11	218 ± 41	474 ± 81
Oxidative stress + E2	78 ± 11[a]	49 ± 6[a]	174 ± 27[a]	332 ± 49[a]
Oxidative stress + E2 + ICI	107 ± 10	62 ± 11	223 ± 46	457 ± 87
Oxidative stress + Progesterone	140 ± 14[a]	86 ± 9[a]	289 ± 40[a]	658 ± 98[a]
Oxidative stress + Progesterone + RU	110 ± 23	75 ± 14	223 ± 32	516 ± 73
Pre-menopausal female controls				
Oxidative stress	91 ± 15	57 ± 9	195 ± 37	435 ± 85
Oxidative stress + E2	64 ± 11[a]	42 ± 6[a]	155 ± 29[a]	314 ± 68[a]
Oxidative stress + E2 + ICI	95 ± 13	54 ± 9	187 ± 37	419 ± 92
Oxidative stress + Progesterone	129 ± 12[a]	75 ± 12[a]	255 ± 40[a]	586 ± 70[a]
Oxidative stress + Progesterone + RU	101 ± 19	63 ± 11	208 ± 40	462 ± 73

The unstimulated PBMCs from the age-matched younger male (n = 18) and pre-menopausal female (n = 18) healthy controls were incubated for up to 6 h in serum-free RPMI after exposure to 10^{-5} mol/L hydrogen peroxide (oxidative stress) with and without 10^{-8} mol/L E2 (E2) or 10^{-7} mol/L progesterone (Progesterone) in the presence and absence of 10^{-6} mol/L ICI (ICI) or 10^{-6} mol/L RU (RU). The levels of TNF-α, IL-1β, IL-8, and MCP-1 in the culture supernatant incubated for 6 h were detected by means of an ELISA. The values are the mean ± SD (n = 18). [a]$P < 0.05$ *vs* 1 h cultures for MCP-1 or 6 h cultures for TNF-α, IL-1β, and IL-8 after hydrogen peroxide exposure in the absence of the female sex hormones and receptor antagonists.

and MCP-1 expressions to be attenuated by E2 in the peritoneal macrophages of female mice[15]. Furthermore, E2 is able to attenuate IL-1β in ER expressing HepG2 cells[16], and to ameliorate the burn-induced increase in the serum TNF-α levels in rats[17]. These findings suggest that E2 may exert a hepatoprotective action against inflammation and oxidative stress, at least in part, by preventing accumulation of monocytes and macrophages and by inhibiting the production of proinflammatory cytokines.

As far as the *in vitro* studies with female sex hormones on the production of TNF-α and IL-1β by monocytes are concerned, however, conflicting data have been published[9], varying from some[18-20] to no[12,21] effect of E2 or progesterone on cytokine production. E2 has been reported to suppress the TNF-α production in unstimulated PBMCs, but not in endotoxin-stimulated PBMCs, from postmenopausal females with osteoporosis[22]. An inhibition of IL-1β production in endotoxin-stimulated monocytes by E2 or progesterone at physiological concentrations has also been reported[23]. The results of the reported studies did not correlate with the present data. These conflicting results may possibly be due to the handling of the cells during the *in vitro* research, different experimental methods used, and/or differences in the subjects employed in the studies.

In the premenopausal female subjects with and without chronic hepatitis C enrolled herein, the blood samples were taken during the luteal phase of the menstrual cycle. During the luteal phase, the serum concentration of endogenous progesterone rises up to a maximum of about 10^{-7} mol/L, which can be ten to a hundred times higher than E2. Higher blood levels of TNF-α have been observed during the luteal phase in comparison to the follicular phase[24]. In males, a higher percentage of IL-1β producing stimulated monocytes has been demonstrated in comparison to females in the follicular phase[19]. The male sex hormone testosterone has some structural and functional similarities to progesterone[25]. Judging from these findings and the present data showing that treatment with E2 (10^{-8} and 10^{-7} mol/L) and progesterone (10^{-7} mol/L) significantly affected the change rate of the cytokine production in hydrogen peroxide-stimulated PBMCs, E2 may, therefore, exert an anti-inflammatory action against both inflammation and oxidative stress in the mononuclear cells from the chronic hepatitis C patients, whereas progesterone may counteract the favorable effects of E2.

HCV infections are recognized to be a major causative factor in the development of liver injury leading to cirrhosis[26,27] The HCV core protein has been reported to enhance the signaling pathway of NF-κB activation in human hepatoma HuH-7 and cervical cancer HeLa cells, and the HCV core protein is triggered by TNF-α-related cytokines[28]. Damage to the parenchymal cell membranes and liver mitochondria could produce ROS derived from lipid peroxidative processes, which constitute a general feature of a sustained inflammatory response and liver injury[29]. In comparison to other types of ROS, hydrogen peroxide is more stable and membrane permeable leading to the hypothesis that it acts as a second messenger in regulating the signaling events, including the mitogen-activated protein kinase (MAPK) activation. We have already reported that E2 inhibited the prooxidant-induced lipid peroxidation in rat liver mitochondria[5], attenuated ROS generation and NF-κB activation in cultured rat hepatocytes in a state of prooxidant-induced oxidative stress[6], while also suppressing the hydrogen peroxide-induced activation of MAPKs and transcription factors including NF-κB in cultured rat hepatic stellate cells[30]. In the present study, hydrogen peroxide exposure resulted in an increase in the TNF-α, IL-1β, IL-8, and MCP-1 levels in the cultured mononuclear cells from the male and female healthy subjects. The oxidative stress-stimulated cytokine expression was attenuated by E2 and augmented by progesterone in a dose-dependent manner without any significant difference between the males and females. These effects of E2 and progesterone were blocked by

their receptor antagonists ICI and RU, indicating that ER and PR could mediate female sex hormone action in the oxidative stress-stimulated monocytes and macrophages.

Finally, the current data suggest that E2 may play a favorable role in the course of persistent liver injury, at least in part, by preventing the accumulation of monocytes and macrophages and by also inhibiting the proinflammatory cytokine production through ER, whereas progesterone may counteract these positive E2 effects by enhancing the accumulation of inflammatory cells and their cytokine production through PR.

COMMENTS

Background

Parenchymal cell membrane damage could produce reactive oxygen species (ROS) derived from lipid peroxidative processes, which represent the general feature of sustained inflammatory response and liver injury. A chronic hepatitis C virus infection tends to progress more rapidly in men than women. There is little information about the effects of estradiol (E2) and progesterone on the proinflammatory cytokine production by prooxidant-stimulated monocytes in chronic hepatic C patients.

Research frontiers

We have recently hypothesized that E2 could play a cytoprotective role in the injured liver by inhibiting the ROS generation, antioxidant enzyme loss, and induction of redox sensitive transcription factors.

Innovations and breakthroughs

This study focused on comparison of the oxidative stress-stimulated proinflammatory cytokine production between younger and older patients, who were divided into two groups based on a mean menopausal age of 50 years, demonstrating that E2 might play a favorable role in the course of persistent liver injury, whereas progesterone might counteract the favorable E2 effects.

Applications

The favorable activity of E2 may be involved in the persistent liver injury of different liver diseases such as alcoholic and non- alcoholic liver diseases as well as chronic hepatitis C and B.

Peer review

The study is of particular interest because the role and importance of the sex hormones in the regulation of some cell functions during disturbance in the prooxidant-antioxidant balance is understood. It is very important that study links clinical and experimental part.

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RAPID COMMUNICATION

Comparison of CT and MRI for presurgical characterization of paraaortic lymph nodes in patients with pancreatico-biliary carcinoma

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Abstract

AIM: To determine the accuracy of computed tomography (CT) and magnetic resonance (MR) for presurgical characterization of paraaortic lymph nodes in patients with pancreatico-biliary carcinoma.

METHODS: Two radiologists independently evaluated CT and MR imaging of 31 patients who had undergone lymphadenectomy (9 metastatic and 22 non-metastatic paraaortic nodes). Receiver operating characteristic (ROC) curve analysis was performed using a five point scale to compare CT with MRI. To re-define the morphologic features of metastatic nodes, we evaluated CT scans from 70 patients with 23 metastatic paraaortic nodes and 47 non-metastatic ones. The short axis diameter, ratio of the short to long axis, shape, and presence of necrosis were compared between metastatic and non-metastatic nodes by independent samples *t*-test and Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS: The mean area under the ROC curve for CT (0.732 and 0.646, respectively) was slightly higher than that for MRI (0.725 and 0.598, respectively) without statistical significance ($P = 0.940$ and 0.716, respectively). The short axis diameter of the metastatic lymph nodes (mean = 9.2 mm) was significantly larger than that of non-metastatic ones (mean = 5.17 mm, $P < 0.05$). Metastatic nodes had more irregular margins (44.4%) and central necrosis (22.2%) than non-metastatic ones (9% and 0%, respectively), with statistical significance ($P < 0.05$).

CONCLUSION: The accuracy of CT scan for the characterization of paraaortic nodes is not different from that of MRI. A short axis-diameter (> 5.3 mm), irregular margin, and presence of central necrosis are the suggestive morphologic features of metastatic paraaortic nodes.

Key words: Paraaortic lymph node; Pancreatico-biliary carcinoma; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Paraaortic lymph node metastasis in the patients with pancreatico-biliary carcinoma has been reported as a definite predictor of early recurrence and shorter survival term, despite differences between individual tumors[1-3]. It is very difficult to preoperatively predict paraaortic node metastasis with imaging, palpation, or intraoperative sonography. Therefore it is recommended that sampling and pathologic confirmation of paraaortic nodes should be performed before starting radical operation. Many surgeons, including

those in our hospital, do paraaortic node dissection before radical surgery[4-7]. Although lymphadenectomy followed by histologic examination of the lymph nodes is still the gold standard for determination of metastasis, this procedure is invasive and could cause many surgical complications[8-11]. Therefore preoperative, noninvasive imaging diagnosis of paraaortic node metastasis is very important[12].

Lymph node staging in the various carcinomas has been extensively discussed in the previous literature[13-19]. Dorfman *et al*[20] reported that the upper limits of the normal nodes in the upper abdomen are site-specific. Therefore, site-specific nodal evaluation is necessary not only due to different clinical importance but also due to different morphologic criteria for malignancy[21]. To our knowledge, however, there have been no radiologic reports on preoperative imaging diagnosis with a focus on the paraaortic node.

The purpose of our study was to compare computed tomography (CT) and magnetic resonance (MR) for preoperative detecting paraaortic lymph node metastasis in the patients with pancreatico-biliary carcinoma and to re-define the significant morphologic features of metastatic ones.

MATERIALS AND METHODS

Patient population

The protocol for this study was approved by the Institutional Review Board at our institution and informed consent for this retrospective study was not required. From February 2000 to June 2006, 70 patients (37 men, 33 women; mean age, 62.9 years) with pancreatic head cancer (n = 22), ampulla of vater cancer (n = 16), distal common bile duct cancer (n = 24), or gallbladder (GB) cancer (n = 8) underwent CT (n = 63) and/or MR (n = 38) imaging. The mean interval time between lymphadenectomy and imaging evaluation was 16.7 d after CT and 18.3 d after MRI. Paraaortic lymphadenectomy was performed in all of the patients before or during surgical resection operations. Histological examinations revealed metastatic paraaortic nodes in 23 patients and non-metastatic nodes in 47 patients. Both CT and MRI were performed in 31 patients with pancreatic head cancer (n = 11), distal common bile duct cancer (n = 13), ampulla of vater cancer (n = 6) or GB cancer (n = 1). Nine patients had metastatic paraaortic nodes and 22 patients had non-metastatic nodes.

Imaging acquisition

All CT scans were obtained with one of the following commercially available multidetector or single detector CT scanners (Somatom Sensation 64, Somatom Sensation 64, Somatom Plus 4; Siemens Medical Solutions, Erlangen, Germany; Lightspeed Plus or QX/i, GE Medical Systems, Milwaukee, Wisconsin). Each patient received 120-150 mL of iopromide (Ultravist 300 or Ultravist 370; Schering, Berlin, Germany) at a rate of 3 mL/s. CT scans were obtained during the arterial phase (using a 25-35-s delay), portal venous phase (using a 70-75-s delay), and equilibrium phase (using a 3-min delay) after IV administration with 3-5-mm section thickness and 3-5-mm reconstruction interval.

MRI examinations were performed using a 1.5-T imaging system (Gyroscan Intera, Philips Medical Systems Best, Netherlands), equipped with commercially available phased-array coils (Synergy; Philips Medical Systems, Best, Netherlands). Four-hour fasting was recommended before the examinations. Antiperistaltic agents or oral contrast agents were not used. The MRI protocol consisted of a breath-hold axial T1-weighted dual fast-gradient-recalled-echo sequence [(TR/in-phase TE, 180/4.6 ms; out-of-phase TE = 2.3 ms; flip angle, 90°; field of view, 32-36 cm × 25-29 cm; matrix, 240 × 240; section thickness, 7 mm; slice spacing, 7.7 mm; one signal acquired; number of slices = 24)]; a single shot turbo spin echo (TR/TE, 452/80 and 160; field of view, 32-36 cm × 25-29 cm; matrix, 288 × 230; section thickness, 7 mm; slice spacing, 5 mm; scan slices were overlapped by 2 mm using an interleaved acquisition technique) with spectral fat suppression and respiratory triggering technique; and a breath-hold transverse 3D gradient echo sequence with fat saturation (TR/TE, 3.9/1.1 msec; flip angle, 25°; field of view, 32-36 cm × 25-36 cm; matrix, 320/224; section thickness, 3 mm).

Contrast-enhanced MRI was performed using a breath-hold 3D gradient echo sequence with fat saturation sequence, following an IV bolus of 0.1 mmol gadobenate dimeglumine (MultiHance, Bracco SpA, Milan, Italy) per kilogram of body weight followed by a saline flush of 30 mL. This sequence was repeated four times with data acquisition in the hepatic arterial, portal venous, and equilibrium phases. An automatic infusion system (Spectris MR injection system, Medrad Europe, Maastricht, Netherlands) operating at an injection rate of 2 mL/s was used. The actual pulse sequence was started manually when the fluoroscopic sequence revealed that the contrast material bolus had reached the abdominal aorta.

Image analysis

All of the imaging analysis was performed on a picture archiving and communication system (PACS) workstation (Centricity 1.0; GE Medical Systems). This retrospective study was composed of two parts. To compare the diagnostic accuracy of CT and MRI, two radiologists independently evaluated preoperative CT and MR images within a 3-wk interval in 31 patients, without knowledge of final pathologic diagnosis. They considered the following criteria as the primary findings for metastatic nodes: (1) short diameter > 9 mm; (2) long axis diameter > 13 mm; (3) presence of necrosis; (4) irregular margin. Reviewers graded the paraaortic lymph node on a five-point scale of diagnostic confidence: 1, no node; 2, definitely benign; 3, probably benign; 4, probably metastatic; and 5, definitely metastatic. Diagnostic accuracy was evaluated using receiver operating characteristic (ROC) curve analysis with a calculation of the area (Az) under the ROC curve. Degree of interobserver agreement was expressed by a Kappa value; a kappa value greater than 0.60 indicated excellent agreement, between 0.40 and 0.60 was good, and less than 0.40 was poor[22].

Using the CT and MR images, we redefined the morphologic criteria of metastatic nodes by comparing them with non-metastatic nodes. Two radiologists evaluated the CT scan in consensus for 63 patients (18 metastatic paraaortic nodes and 45 non-metastatic ones)

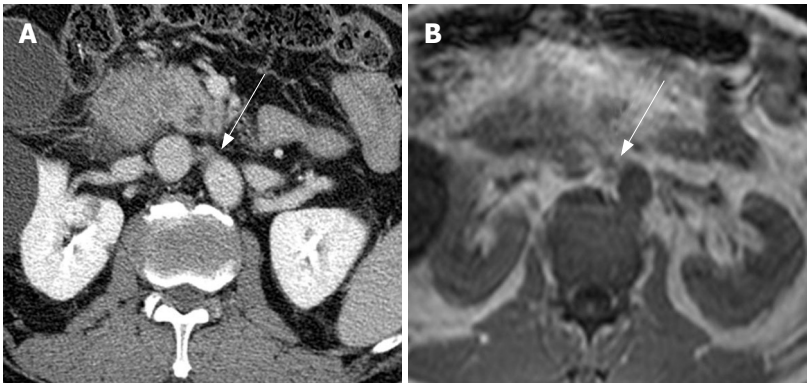

Figure 1 Metastatic right paraaortic lymph node in a 63-year-old man with pancreatic head cancer. **A**: Contrast-enhanced CT shows an irregularly shaped lymph node (arrow) with a short axis dimension of 11.5 mm that was interpreted as a definitely metastatic lymph node; **B**: Axial T1-weighted MRI shows an irregularly shaped lymph node (arrow) with a short axis dimension of 8.5 mm that was interpreted as a definitely benign lymph node. Pathologic examination revealed that this lymph node was metastatic.

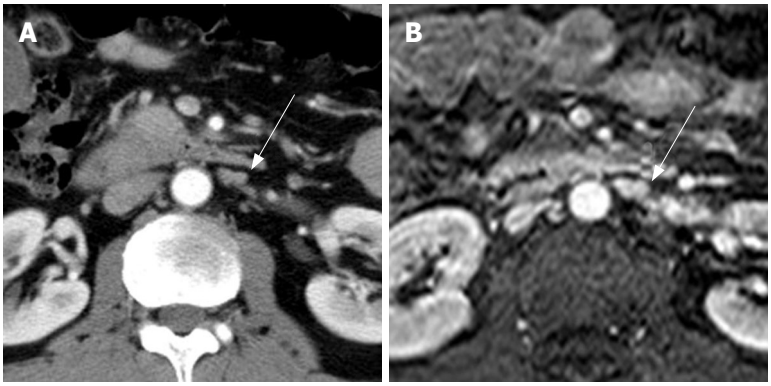

Figure 2 Metastatic left paraaortic lymph node in a 51-year-old man with pancreatic head cancer. **A**: Contrast-enhanced CT shows an irregularly shaped lymph node (arrow) with a short axis dimension of 7.2 mm that was interpreted as a probably metastatic lymph node; **B**: Axial contrast-enhanced T1-weighted MRI shows an irregularly shaped lymph node (arrow) with a short axis dimension of 7 mm that was interpreted as a probably metastatic lymph node; this diagnosis was confirmed by lymphadenectomy and pathological examination.

to record the short and long axial diameter and their ratio, margin (smooth or irregular), and the presence of necrosis in the detected paraaortic lymph nodes. The short and long axis diameter and their ratio were compared between metastatic paraaortic and non-metastatic lymph nodes by the independent samples *t*-test. The margin and presence of necrosis of metastatic paraaortic lymph nodes were compared to those of non-metastatic nodes by Fisher's exact test. $P < 0.05$ was considered statistically significant. A ROC curve was used to determine the best cut-off value for the short and long axis diameter for differentiation of metastatic from non-metastatic nodes. When multiple nodes in the paraaortic region were detected, the largest, irregular-shaped, and/or necrotic node was selected and defined as a metastatic node. The imaging findings were compared with histopathologic results on a per-case basis.

RESULTS

Accuracy of CT and MRI for detecting metastatic paraaortic lymph nodes

Interobserver agreement between the two readers for CT was excellent (kappa value 0.674; standard error 0.088), but that for MRI was poor (kappa value 0.359 ; standard error 0.157).

The mean area under the two readers' ROC curve for CT (0.732 and 0.646, respectively) was slightly higher than that for MRI (0.725 and 0.598, respectively) without statistical significance ($P = 0.940$ and 0.716, respectively) (Figures 1 and 2).

Features of metastatic paraaortic lymph nodes on CT

The comparison between non-metastatic and metastatic paraaortic lymph nodes on CT is summarized in Table 1. The short axis diameter of metastatic lymph nodes (mean = 9.2 mm, 3.8-28.1 mm) was significantly larger than that of non-metastatic lymph nodes (mean = 5.17 mm, 2.1-11.8 mm, $P < 0.05$). The long axis diameter of metastatic lymph nodes (mean = 13.18 mm, 5-32.1 mm) was significantly larger than that of non-metastatic lymph nodes (mean = 8.72 mm, 4.6-22.9 mm, $P < 0.05$). However, the ratio of the short to long axis of metastatic lymph nodes (mean = 0.70) was slightly larger than that of non-metastatic lymph nodes (mean = 0.58) without statistical significance ($P = 0.284$). The margins of the paraaortic lymph nodes were irregular in 8 of 18 patients (44.4%) with metastasis (Figures 1 and 2), and 4 of 45 patients (8.9%) without metastasis. The presence of central necrosis was seen in 4 of 18 patients (22.2%) with metastasis, but was not seen in patients without metastasis. Metastatic nodes had more irregular margins (44%) and central necrosis (28%) than non-metastatic nodes (9% and 0%, respectively), with statistical significance ($P < 0.05$).

Table 1 Features of metastatic paraaortic lymph nodes on CT

	Non-metastatic	Metastatic	*P* value
Mean short diameter	5.17 mm	9.2 mm	< 0.05
Mean long diameter	8.72 mm	13.18 mm	< 0.05
Mean ratio (short/long)	0.58	0.7	0.284
Irregular margin	9%	44%	< 0.05
Necrosis	0%	28%	< 0.05

Based on the ROC curve, we determined that the best cut-off values for differentiating metastatic nodes from non-metastatic nodes were > 5.3 mm for the short axis diameter

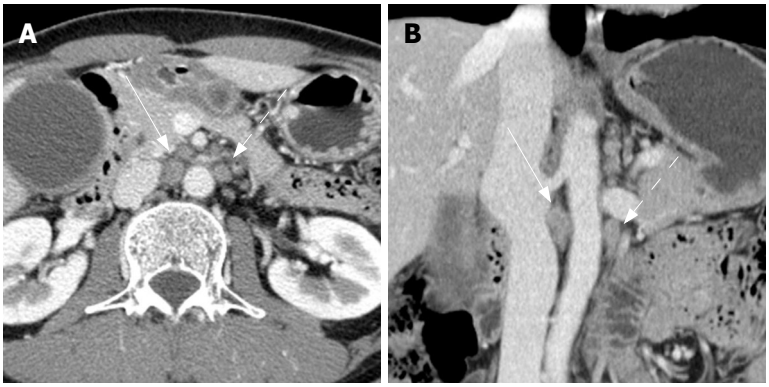

Figure 3 Two metastatic paraaortic lymph nodes in a 49-year-old man with gallbladder cancer. Axial (**A**) and coronal (**B**) contrast-enhanced CT shows several paraaortic lymph nodes. Among them, the right largest node (straight arrow) shows 10 mm and 18.8 mm of short and long axis diameters with irregular margin (on coronal image), compatible with metastatic node. The left one (dot arrow) shows 8.2 mm and 12.2 mm of short and long axis diameters, less than the mean value of metastatic ones (9.2 mm and 13.2 mm, respectively). According to the best cut-off value of short diameter more than 5.3 mm and long axis diameter more than 11.6 mm, The left one is also metastatic one rather than non-metastatic one. Pathologic examination revealed that two lymph nodes were metastatic ones among six resected paraaortic lymph nodes.

and > 11.6 mm for the long axis diameter (Figure 3). According to the short axis cutoff of > 5.3 mm, the diagnostic values for metastatic nodes were 77.8% sensitivity (95% confidence interval (CI): 52.4%-93.5%) and 66.4% specificity (95% CI: 48.8%-78.1%). According to the cutoff of > 11.6 mm for the long axis diameter, the diagnostic values for metastatic nodes were 50.0% sensitivity (95% CI: 26.1%-73.9%) and 91.1% specificity (95% CI: 78.8%-97.5%).

DISCUSSION

Although CT and MR imaging are well established for the staging and follow-up of patients with malignancy, the rates of accuracy for the detection of metastatic lymph nodes vary widely. It has been reported that the accuracy of CT and MRI for the detection of lymph nodes in patients with cervical carcinoma[23-25] and the evaluation of regional nodes in the patients with rectal cancer[26,27] is comparable. Other studies have suggested that CT is more specific for detecting positive lymph nodes in gynecologic cancers, whereas MR imaging is more sensitive[23]. In some reports on the evaluation of cervical cancer, MRI (60%) was reported to be more sensitive than CT (43%), whereas the specificities of the two modalities were comparable[28]. Focusing on paraaortic nodes in the patients with pancreatico-biliary cancer, our study showed that the accuracy of CT and MRI were comparable. Our results revealed that the interobserver agreement for CT was excellent, whereas that for MR was poor. This finding suggests that the radiologist's experience is more important for evaluating by MRI than CT, although it is generally accepted that the tissue contrast with MRI is better than that with CT.

Size criteria have been used in the differentiation of metastatic from non-metastatic nodes, despite much dispute[29]. In past, the maximum short axis diameter of a normal lymph node was known to vary on abdominal computed tomography, according to the node's location; the upper paraaortic region is 9 mm and the lower paraaortic region is 11 mm[20]. A recent study for metastatic paraaortic nodes in pancreatic cancer shows that the size criteria combined with a long axis diameter (12, 10, 8, or 6 mm) and the axial ratio (0.5, 0.7, or 1.0) have a positive predictive value of 13% to 36% and an overall accuracy of 66.7% to 78.9%[21]. Therefore it has been concluded that morphologic criteria are not useful in the evaluation of metastatic paraaortic nodes. A previous study of gallbladder carcinoma, on the other hand, demonstrated a high positive predictive value (86%) in the evaluation of metastatic interaortocaval nodes based on the size and shape criteria; anterior posterior dimensions of 10 mm or larger and ring-like or heterogeneous contrast enhancement[30]. In our study, there was a statistically significant difference between two groups: the mean values for the short and long axis diameter of metastatic paraaortic nodes were 9.2 mm and 13.18 mm, respectively, whereas those of nonmetastatic ones were 5.17 mm and 8.27 mm, respectively. In our study, the best cut-off value for differentiating metastatic nodes from non-metastatic nodes was a short axis diameter of more than 5.3 mm (77.8% sensitivity and 66.4% specificity) and a long axis diameter of more than 11.6 mm (50.0% sensitivity and 91.1% specificity).

It is well known that central necrosis has a very high positive predictive value (almost 100%) in the diagnosis of metastasis. Our study also demonstrated that central necrosis was seen only in metastatic nodes. However, central necrosis may be seen with tuberculosis. Moreover, the sensitivity of central necrosis is very low. In our study, irregular margin had a high positive predictive value, although it was not pathognomic.

Our study had some limitations. First, it was a retrospective study and the parameters of the CT and MRI were not uniform. Second, the imaging findings were compared with histopathologic results on a per-case basis not on a per-node basis.

In conclusion, we found that the accuracy of CT and MRI were comparable for the evaluation of paraaortic nodes in the patients with pancreatico-biliary cancer. Central necrosis, irregular margin, and a cut-off value of more than 5.3 mm for the short axis diameter and 11.6 mm for the long axis diameter may be used as the criteria for diagnosing metastatic paraaortic nodes on CT scan. However, functional studies, such as high-resolution MRI with lymphotropic contrast agent, are necessary to overcome the limitation of morphologic evaluation of nodes.

COMMENTS

Background

In patients with pancreatico-biliary carcinoma, paraaortic lymph node metastasis has a crucial impact on surgical indication or extent of operation. At present, many surgeons perform paraaortic lymphadenectomy for accurate assessment and

decision for adequate extent of operation. However, because of its invasiveness and complications, paraaortic lymphadenectomy for pancreatico-biliary carcinoma is controversial.

Research frontiers

Although a comparison between computed tomography (CT) and magnetic resonance (MR) has already been performed in cervical cancer, colorectal cancer and other malignancy, no studies to date have compared CT with MR in terms of detecting paraaortic lymph node metastases from pancreatico-biliary carcinoma. The aim of this study is to determine the accuracy of CT and MR for presurgical characterization of paraaortic lymph nodes in patients with pancreatico-biliary carcinoma.

Innovations and breakthroughs

The results of this study indicate that the accuracy of CT and MR were comparable for the evaluation of paraaortic nodes in the patients with pancreatico-biliary cancer. The lymph node diameter > 5.3 mm, irregular margin, and central necrosis are the suggestive morphologic features of metastatic paraaortic nodes.

Applications

CT and MR could be used for the selection of candidates for lymphadenectomy in the patients with pancreatico-biliary carcinoma.

Terminology

Paraaortic lymph node metastasis in the patients with pancreatico-biliary carcinoma has been reported as a definite predictor of early recurrence and shorter survival term.

Peer review

This is a very interesting paper, although it is a retrospective study. The idea of paraaortic lymph node in pancreatico-biliary is important for evaluation. This unique study will be a first step to confirm the results of a prospective study in the future.

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RAPID COMMUNICATION

Thrombospondin-1 expression correlates with angiogenesis in experimental cirrhosis

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Abstract

AIM: To investigate the significance of Thrombospondin-1 (TSP-1) expression and its relationship with angiogenesis during experimental fibrosis.

METHODS: Cirrhosis was induced in male Wistar rats by intraperitoneal administration of diethyl nitrosamine (DEN). The serial sections from liver tissues were stained with anti-CD34 and anti-TSP-1 antibodies before being quantitated by light microscopy.

RESULTS: Our results showed that of TSP-1 expression gradually increases according to the severity of fibrosis (Group Ⅰ *vs* group Ⅱ, Group Ⅲ and Group Ⅳ; Group Ⅱ *vs* group Ⅲ and group Ⅳ; group Ⅲ *vs* group Ⅳ, $P < 0.05$). Moreover, TSP-1 expression was found to be correlated with angiogenesis ($P < 0.05$).

CONCLUSION: The correlative evidence of the link between TSP-1 and fibrosis or angiogenesis provided by this study suggests that besides its role as a strong promoter of transforming growth factor-β1 (TGF-β1), TSP-1 might have an additional role in liver fibrogenesis by stimulating angiogenesis and this protein could be a potential target to prevent fibrogenesis in chronic inflammatory diseases of the liver.

Key words: Experimental liver cirrhosis; Immunohistochemistry; Liver fibrosis; Pathologic angiogenesis; Thrombospondin-1

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INTRODUCTION

Hepatic angiogenesis is frequently associated with inflammation and fibrogenesis during chronic liver injury[1-5]. Currently, it is not clear whether this process plays a beneficial role in the maintenance of homeostasis or contributes to liver damage during chronic inflammation. However, the fact that chronic inflammatory liver diseases respond poorly to immunosuppressive and anti-inflammatory therapy suggests that angiogenesis might be a promising therapeutic target in the prevention of fibrosis[6-8]. For this reason, attempts are being directed to evaluate the cellular and molecular mechanisms involved in the development of hepatic angiogenesis during chronic liver injury[3-5].

Thrombospondin 1 (TSP-1), one of the five members of the Thrombospondin gene family, is a matrix protein involved in complex processes including wound healing and angiogenesis[9,10]. The exact role of TSP-1 in angiogenesis is still controversial. TSP-1 can function as an inhibitor or as a promoter of angiogenesis, indicating that it might modulate this process in opposite directions[11-16]. In malignant and premalignant conditions of the liver, TSP-1 expression and its association with angiogenesis have been demonstrated[15-18]. Regarding non-neoplastic liver diseases, although the association of TSP-1 with latent transforming growth factor-β1 (TGF-β1) has been demonstrated in a few studies, the relationship between TSP-1 and angiogenesis during liver fibrogenesis has not been documented[19-21].

Therefore, this study was undertaken to investigate the significance of TSP-1 expression during diethyl nitrosamine (DEN) induced experimental liver fibrosis and to evaluate whether any relationship exists between TSP-1 expression and angiogenesis.

MATERIALS AND METHODS

Materials

This animal study was approved by the local animal ethics committee of the Akdeniz University. Male adult Wistar rats weighing 250 g were used. They were maintained on

Table 1 Distribution of mean, standard deviation, median and ranges of VD and TSP-1 expression in normal livers (group Ⅰ) and in DEN treated livers (group Ⅱ, group Ⅲ, and group Ⅳ)

Group	VD[a]			TSP-1 expression (%)[a]		
	mean ± SD	Median	Ranges	mean ± SD	Median	Ranges
Ⅰ (*n*: 8)	3.24 ± 1.41	3	2-6	1.63 ± 1.06	1.5	0-3
Ⅱ (*n*: 9)	5.22 ± 1.86	5	2-8	5.89 ± 1.18	4	0-14
Ⅲ (*n*: 10)	9 ± 4.57	6	1-16	16.3 ± 7.32	9	2-26
Ⅳ (*n*: 10)	14.5 ± 5.97	11	8-26	68.5 ± 19.73	44	0-95

n: Number of cases; VD: Vascular density; SD: Standard deviation; [a]$P < 0.05$.

a commercial diet and water in a room at 22 ± 2℃ under normal laboratory lighting conditions.

Methods

Animal model: The rats received intra-peritoneal injections of DEN (Sigma, Saint Quentin Fallavier, France) at 100 mg/kg of body weight (*n*: 29) or 0.9% sodium chloride (*n*: 8) once a week. The injections were performed for 2 (*n*: 4), 4 (*n*: 5), 5 (*n*: 5), 6 (*n*: 5), 8 (*n*: 5) and 10 (*n*: 5) wk. The animals were sacrificed 2 wk after the last administrations and a hepatectomy was performed. Liver tissue samples were either frozen immediately in liquid nitrogen and stored at -70℃ or fixed in 10% buffered formalin and embedded in paraffin.

Histology and immunohistochemistry: Four micrometer thick serial sections from the liver tissues originally fixed in formalin and embedded in paraffin were prepared and stained with hematoxylin and eosin for the histopathological assessment. Masson trichrome staining was used in the evaluation of the extent of liver fibrosis.

Immunolabeling was performed using polyclonal antibodies directed anti rat CD34 (sc- 7045 goat, dilution: 1:500, Santa Cruz, CA, USA) and thrombospondin-1 (sc-12312 goat, IgG, dilution 1:200, Santa Cruz, CA, USA). An avidin-biotin-peroxidase technique (sc-2023, anti-goat ABC staining Kit; Santa Cruz, CA, USA) was used for labeling. For CD34, sections from paraffin embedded tissue blocks were dried in a hot air oven at 55℃ overnight and dewaxed. Microwave antigen retrieval (750 W, 4 × 5 min in citrate buffer 0.01 mol/L, pH 6) was performed. TSP-1 staining was applied to 5 μm thick air dried (30 min) cryostat sections, fixed in acetone (10 min). Endogenous peroxidase was blocked by using 3% hydrogen peroxide in methanol for 30 min. Each step of incubation was followed thorough washing of the slides in phosphate buffered saline (PBS). After incubation with primary antibody against CD34 and TSP-1 (each 30 min), sections were reacted with secondary biotinylated antibody (30 min) and AB enzyme reagent (avidin and biotinylated horseradish peroxidase) for 30 min. Finally, all slides were treated with DAB reagent to develop color and counterstained with Mayer's hematoxylin. Normal goat and rabbit IgG instead of primary antibodies were used as negative control at the same dilution.

The vascular density in portal and periportal areas was assessed by determining the count of CD34 labeled vessel sections at higher magnification (× 400) with the use of an ocular grid subdivided into 100 areas. For each subject vascular density (VD) was noted.

For quantitative evaluation of TSP-1 expression, in each section positive and negative cells were counted in systematically randomly selected 10 to 15 microscopic fields by using an ocular grid at high magnification (× 400). The positive staining was calculated as the percentage of positive cells to total number of counted cells. Positive cells touching the left and lower edge of the grid were not included.

All analysis were performed using Statistical Package for Social Science (SPSS 15.0 for Windows, USA). Mann-Whitney-*U* test was used to establish the difference between groups. Friedman test was used to determine the relationship between quantitative parameters. Data were expressed as mean ± SD and $P < 0.05$ was considered significant.

RESULTS

In this study, fibrogenesis was not observed in the control group. In DEN treated rats, fibrous septa were detected after 5 wk. The liver was cirrhotic in all cases after 8 wk. According to the severity of fibrosis, cases were divided in following groups: Group Ⅰ: normal livers, group Ⅱ: non-fibrotic livers (2 and 4 wk), group Ⅲ: fibrotic livers (5 and 6 wk) and group Ⅳ: cirrhotic livers (8 and 10 wk) (Figure 1A). In group Ⅰ, CD34 staining was restricted to the endothelium of portal vessels. While in non-fibrotic livers CD34 expression was noted in a few vascular structures around portal areas, numerous CD34-labeled vessels were detected in fibrotic livers. In group Ⅳ, CD34 staining revealed a dense vascular plexus surrounding the cirrhotic nodules (Figure 1B). Parallel to this finding, VD values were increased together with the progression of fibrosis (Figure 2). DEN-treated cases (group Ⅱ, Ⅲ and Ⅳ) had higher VD than the control group ($P < 0.05$). The difference between VD values of group Ⅱ, Ⅲ and Ⅳ was also statistically significant ($P < 0.05$) (Figure 2 and Table 1).

In normal livers (group Ⅰ), TSP-1 expression was restricted to the endothelium of portal vessels and to a few hepatocytes (Figure 1C). However, in non-fibrotic group TSP-1 expression was higher than normal livers with more positive hepatocytes and perisinusoidal cells ($P < 0.05$). TSP-1 expression continued to increase in fibrotic livers and was more widespread in cirrhotic livers. The expression of TSP-1 in DEN-treated rat groups was significantly different from each other ($P < 0.05$) (Figure 1C, Figure 2 and Table 1).

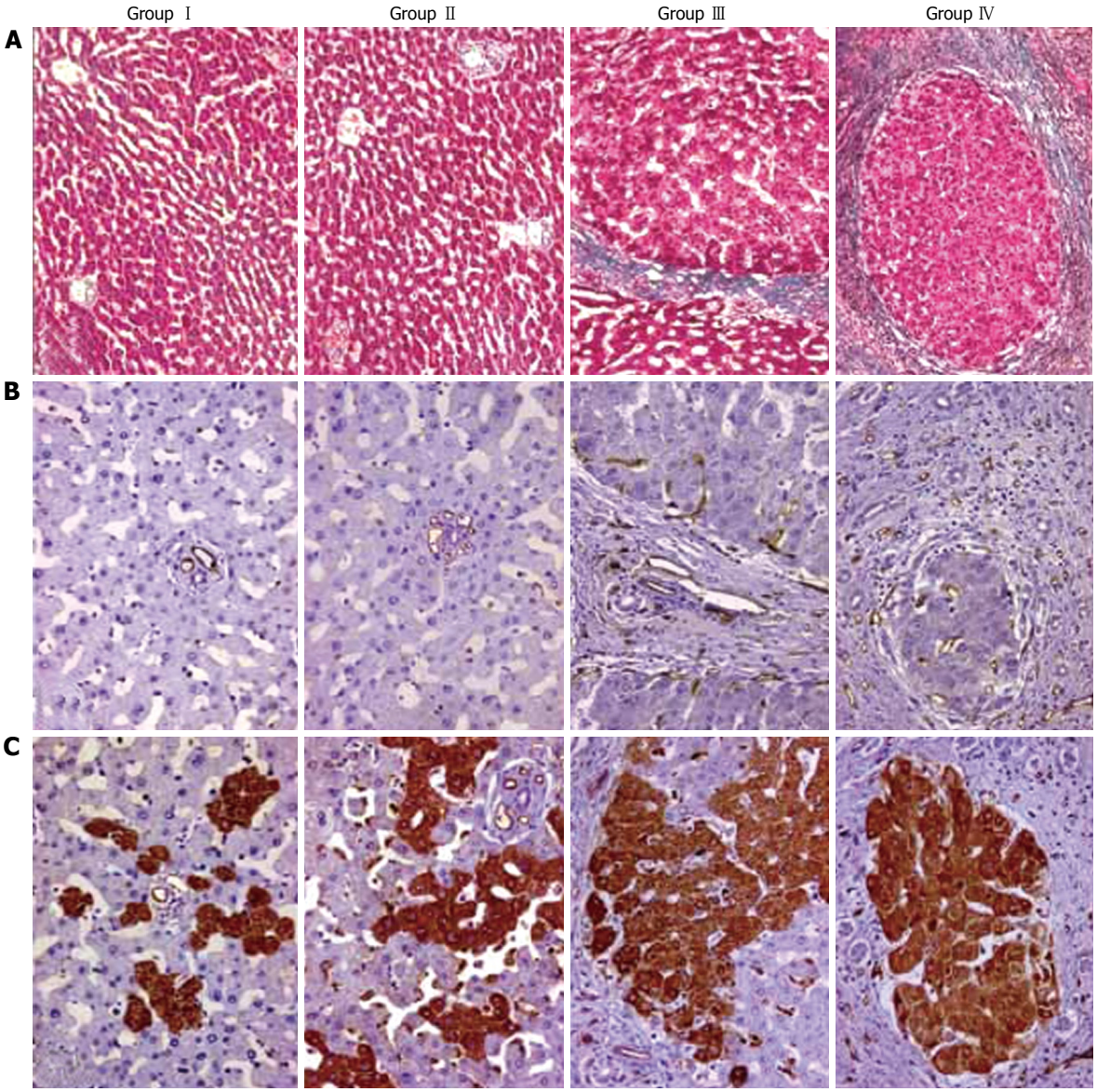

Figure 1 Liver fibrosis (**A**), angiogenesis (**B**) and TSP-1 expression (**C**) in the study group. Liver fibrosis was stained by Masson trichrome at different time points of treatment and angiogenesis was evaluated with an anti-CD34 antibody. In normal livers, the number CD34 labeled vessels and TSP-1 positive cells is lower when compared to DEN treated livers. In the latter, their number increases according to the extent of fibrosis.

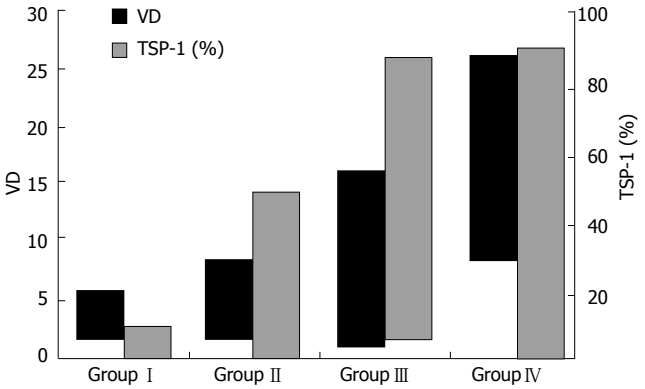

Figure 2 Results of the quantitative assessment of angiogenesis and TSP-1 expression in normal and DEN treated rat livers. There is a gradual increase for VD and TSP-1 expressions parallel to the severity of fibrosis.

Friedman test showed that there was a significant correlation between VD and TSP-1 expression ($P < 0.05$).

DISCUSSION

Results of the recent studies emphasized that hepatic angiogenesis is associated with fibrogenesis in the wound healing response to chronic liver injury[1-5]. In our study, parallel to this finding, angiogenesis, assessed as VD, was increased with the progression of fibrosis ($P < 0.05$). Besides, in group Ⅱ, despite the absence of overt fibrosis, VD was higher than that of normal livers, suggesting that angiogenesis is an early event which might take place before the onset of fibrosis during chronic liver damage.

It is well known that angiogenesis does not involve a single pathway, but is a complex event regulated by many angiogenic and antiangiogenic factors, including TSP-1[9,10]. In neoplastic and premalignant conditions of the liver, the relationship between TSP-1 expression and angiogenesis has been studied[15-17]. However, in non-neoplastic liver diseases the association of TSP-1 expression with angiogenesis and its role in this complex event has not

been documented. Because TSP-1 is also a known activator of TGF-β1, a key mediator in tissue fibrogenesis, a few studies has been focused to evaluate the effect of TSP-1 in hepatic activation of TGF-β1[19-21]. It was concluded that TSP-1 may act in the pathogenesis of liver fibrogenesis as a strong promoter of TGF-β1. Although in the present study TGF-β1 expression was not evaluated, we observed an increase of TSP-1 expression parallel to the severity of fibrosis. TSP-1 expression of normal livers was restricted to the endothelium of portal vessels and to a few hepatocytes. However, this value was 3.61 fold higher in non-fibrotic group. The percentage of TSP-1 expressing cells continued to increase in fibrotic and cirrhotic livers ($P < 0.05$). The present data support the contribution of TSP-1 expression in the wound healing response to chronic liver injury[19-21]. Moreover, in this study, a strong correlation between TSP-1 expression and angiogenesis was observed ($P < 0.05$). This finding suggests that TSP-1 is not only involved in fibrogenesis by the hepatic activation of TGF-β1 but also might play another role in the remodeling of the liver architecture by contributing to the development of angiogenesis.

Our results showed TSP-1 might be a stimulator of angiogenesis during liver injury. TSP-1 is generally recognized as an antiangiogenic agent[11,12,15]. However results of the some studies have been demonstrated that TSP-1 might be a stimulator of angiogenesis[13,14-16]. For this reason the actual role played by TSP-1 in angiogenesis has been investigated in previous studies with several different conclusions. Some studies demonstrated that the effect of TSP-1 may depend on its concentration[13,22], the type of domain being activated[23] and the type of receptors present on endothelial cells[24]. It has been also speculated that the actual role of TSP-1 might be related to number of its receptors[25]. Although it is not possible to conclude, based on our findings, which factor determines the angiogenic effect of TSP-1 during chronic liver injury, our data reinforce its dual role in the modulation of angiogenesis in opposite directions.

In conclusion, the results of this descriptive study reveal that in experimental liver fibrogenesis TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis. Our data suggest that TSP-1 might contribute to the wound healing response to liver injury not only as a strong promoter of TGF-β1, but also as an inducer of angiogenesis. In the light of this observation, it would be of interest to evaluate the mechanism triggered by TSP-1 in hepatic angiogenesis with further experimental models, in order to completely clarify if TSP-1 could be a potential target in the manipulation of angiogenesis in chronic inflammatory liver diseases ending with cirrhosis.

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COMMENTS

Background

Angiogenesis progresses together with fibrogenesis in the wound healing response to chronic liver injury. Thrombospondin-1 (TSP-1) is a matricellular protein which is involved in complex processes including wound healing and angiogenesis. TSP-1 might modulate angiogenesis in opposite directions. In malignant and premalignant conditions of the liver the relationship between TSP-1 expression and angiogenesis have been demonstrated. Regarding non-neoplastic liver diseases, although the association of TSP-1 with latent transforming growth factor-β1 (TGF-β1) has been demonstrated in a few studies, the relationship between TSP-1 and angiogenesis during liver fibrogenesis has not been documented.

Research frontiers

At present it is not possible to ascertain the exact pathogenic role of angiogenesis in liver fibrogenesis. However, the fact that chronic liver diseases respond poorly to conventional therapies suggests that manipulation of angiogenesis could be a promising approach to treatment. For this reason, the cellular and molecular mechanisms that are involved in the development of angiogenesis during liver fibrogenesis have been a topic of intensive investigations in the recent years.

Innovations and breakthroughs

This study demonstrated that in experimental liver fibrogenesis TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis.

Applications

Based on the results of this research, TSP-1 might contribute to the wound healing response to liver injury not only as a strong promoter of TGF-β1, but also as an inducer of angiogenesis and could be a potential target in the manipulation of angiogenesis in chronic inflammatory liver diseases ending with cirrhosis.

Terminology

TSP-1 is a high molecular weight glycoprotein (450 kDa) which is composed of three identical subunits cross-linked by disulfide bonds. Each subunit is composed of several domains interacting with different surface receptors. TSP-1 is involved in various processes such as cell motility, inflammation and wound healing. It also modulates endothelial cell adhesion, motility and growth.

Peer review

This is quite an interesting investigational paper. This study demonstrated that in experimental liver fibrogenesis, TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis. Further study would focus on evaluating the mechanism of TSP-1 in hepatic angiogenesis.

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RAPID COMMUNICATION

Sustained virological response based on rapid virological response in genotype-3 chronic hepatitis C treated with standard interferon in the Pakistani population

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Abstract

AIM: To document the sustained virological response (SVR) in rapid virological responders (RVR) of genotype-3 chronic hepatitis C with standard interferon (SdIF).

METHODS: Hepatitis C genotype-3 patients during the period July 2006 and June 2007 were included. Complete blood counts, prothrombin time, ALT, albumin, qualitative HCV RNA were done. SdIF and ribavirin were given for 4 wk and qualitative HCV RNA was repeated. Those testing negative were allocated to group-A while the rest were allocated to group-B. Treatment was continued a total of 16 and 24 wk for group A and B respectively. HCV RNA was repeated after 24 wk of treatment. End virological and sustained virological responses were compared by χ^2 test. ROC of pretreatment age, ALT and albumin were plotted for failure to achieve SVR.

RESULTS: Of 74 patients treated, RCV RNA after 16 wk of therapy became undetectable in 34 (45.9%) and was detectable in 40 (54.1%) and were allocated to groups A and B respectively. SVR was achieved in 58.8% and 27.8% in groups A and B respectively. SVR rates were significantly higher in patients who had RVR as compared to those who did not (P = 0.0; γ = 2). Both groups combined ETR and SVR were 70% and 33% respectively. ROC plots of pretreatment age, ALT and albumin for SVR showed only ALT to have a significantly large area under the curve.

CONCLUSION: SVR rates were higher in patients who had RVR with SdIF and high pre treatment ALT values correlated to probability of having RVR.

Key words: Hepatitis C; Sustained virological response; Rapid virological responders; Chronic hepatitis

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INTRODUCTION

Hepatitis C virus (HCV) is a big health care problem all over the world with 130 million patients infected with this virus world over[1,2]. The problem in developing countries is compounded by the poor economical status of the patients who are unable to afford the expensive therapy. The most prevalent genotype in Pakistan is type-3 (68%-87%) which has a favorable response to standard interferon[3-6]. Recently some studies have suggested 16 wk therapy with pegylated interferon (PgIF) in patients who achieve rapid virological response (RVR) at 4 wk. The SVR rates in patients with RVR with pegylated interferon have been reported at 78% and that with standard interferon (SdIF) at 53%[7]. The treatment protocols that consider viral load and RVR are proven to be more cost effective than standard protocols[8]. For genotype-2 most of the studies are in favour of a 16 wk therapy in patients who achieve RVR but there is no such recommendation for genotype-3 and more data is required to make any such recommendation[9].

In a poor country like Pakistan affordability of even 16 wk of PgIF treatment is financially difficult. There is

no report of 16 wk standard interferon therapy results regarding sustained virological response (SVR) from Pakistan. The current study was conducted to see the end treatment response (ETR) and SVR in patients who achieved the RVR with SdIF.

MATERIALS AND METHODS

Subjects

This interventional study was conducted at Civil Hospital and Anklesaria Nursing Home Karachi during the period July 2006 and June 2007. Naïve patients of chronic HCV of Genotype-3 were included. Patients with decompensated disease, depression, allergy to interferon were excluded. Complete blood counts (CBC), prothrombin time (PT), ALT, albumin, qualitative HCV RNA and genotype were done before start of therapy. Therapy was started with SdIF α 2a 3.0 MU thrice weekly (TIW) and ribavirin 800-1200 mg PO according to weight. Qualitative HCV RNA was repeated after 4 wk into therapy. Patients who tested negative at this stage were allocated to Group-A while those in which it was still detectable were allocated to Group-B. Patients in Group-A were continued with the same therapy for 12 more wk for a total of 16 wk and then the therapy was stopped. These patients were retested for HCV RNA after 24 wk of stoppage of therapy for SVR. Patients in Group-B were continued with the same therapy for a total of 24 wk and were retested for HCV RNA for ETR. Patients in which HCV RNA was still detected after 24 wk of therapy were labeled as non-responders. Those who tested negative were followed for a further 24 wk without any treatment and retested for HCV RNA for SVR (Figure 1).

Methods

CBC was done by a Sysmex Autoanalyzer while biochemical tests were done by Hitachi Autoanlyzer using Merck biomedical reagents. HCV RNA was done by Roche reverse transcriptase method. Sample size was determined for hypothesis testing for two population proportions[10]. Keeping the level of significance at 5%, power of study at 90% and reported SVR rates of 78% and 53% the sample size was calculated to be of 74 patients[7]. Scale variables of age, ALT, hemoglobin, albumin were compared between the two groups by Students *t*-test. The nominal variables of gender, ETR & SVR were compared by χ^2 test. Receiver operator curve (ROC) was plotted for age, ALT and albumin at the start of treatment. Failure to achieve RVR was taken as a variable state.

Statistical analysis

The significant level was set at $\leqslant 0.05$. SPSS version 15.0 was used[11].

RESULTS

Seventy four eligible patients of genotype-3 HCV satisfying the selection criteria were included. These included 50 (67.6%) males and 24 (32.4%) females. Mean age of males was 35.9 ± 8.0 years and that of females was 39.1 ± 8.1 years. No statistically significant difference was found between the ages of the two genders ($P = 0.11$; $\gamma = 72$; 95% CI = -7.7 to 0.8) (Table 1). Patients were started with SdIF 3.0 MU TIW SQ with ribavarin according to body mass. RVR was achieved in 34 (45.9%) of the patients. These patients were allocated to Group-A while the rest of 40 (54.1%) patients who didn't achieve RVR were allocated to Group-B. Patients in Group-A were continued with the same treatment for a further 12 wk making a total of 16 wk of therapy. They were followed off treatment for a period of 24 wk and HCV RNA was repeated for SVR. Among these 20 (58.8%) patients achieved the SVR while 14 (41.2%) had a relapse within 24 wk of the follow-up period. In Group-B after 24 wk of therapy the ETR was achieved in 18 patients while 22 patients did not respond and were excluded from further analysis. After the off treatment follow-up of 24 wk in patients who achieved the ETR in group-B, the SVR was present in 5 (27.8%) and relapse was detected in 13 (72.2%) patients. Comparing the SVR rates between the two groups, SVR rates were statistically higher in Group-A ($P = 0.044$; $\gamma = 1$). SVR rates were significantly higher in patients who had RVR as compared to those who didn't; 20/34 (58.8%) *vs* 5/40 (12.5%) ($P = 0.0$; $\gamma = 2$). The combined outcome results show that RVR was achieved in 34 (45.9%), ETR in 52 (70.3%) & SVR in 25 (33.8%). The total relapse rate was 27 (36.5%) while the total non-responder rate was 22 (29.7%).

Table 1 Demographic details of studied population

	Group A (n = 34)	Group B (n = 40)	γ	*P*	95% CI
Age (yr)	35.7 ± 8.2	38.0 ± 8.0	72	0.110	-7.198 to 0.751
M:F	22:12	28:12	1	0.804	-
ALT (U/L)	117.5 ± 38.4	160.4 ± 38.6	72	0.000	-60.770 to -24.971
Albumin (g/L)	38 ± 7	38 ± 4	72	0.789	-0.312 to 0.238

ROC plots for age, ALT, and albumin at the time of induction were plotted for failure to achieve RVR at the end of 4 wk (Figure 2). The area under the curve for ALT was significantly high at 0.8 with $P = 0.000$. This shows that among the three variables ALT had strong predictive value for RVR failure. At the cutoff of 73.5 IU/dL the sensitivity and specificity of ALT for RVR failure was 1.0 and 0.94 respectively. Thus values of ALT $\leqslant$ 73.5 U/L at the start of treatment were less likely to have RVR.

DISCUSSION

Although much research has gone into the treatment of chronic HCV, the optimal treatment is not yet established[12]. Many treatment options are in vogue with different success rates[13-15]. PgIF based protocols have better responses as compared to the SdIF but cost becomes the major hurdle in developing countries. In Pakistan the most common genotype is 3 which is highly responsive to SdIF[5,16]. RVR is now a new landmark in the treatment and retreatment of HCV and it not only determines the duration but also predicts the outcome of the therapy[17-19]. For genotype-2 most of the studies are in favour of a 16 wk therapy in patients who achieve RVR but there is no such recommendation for genotype-3[9].

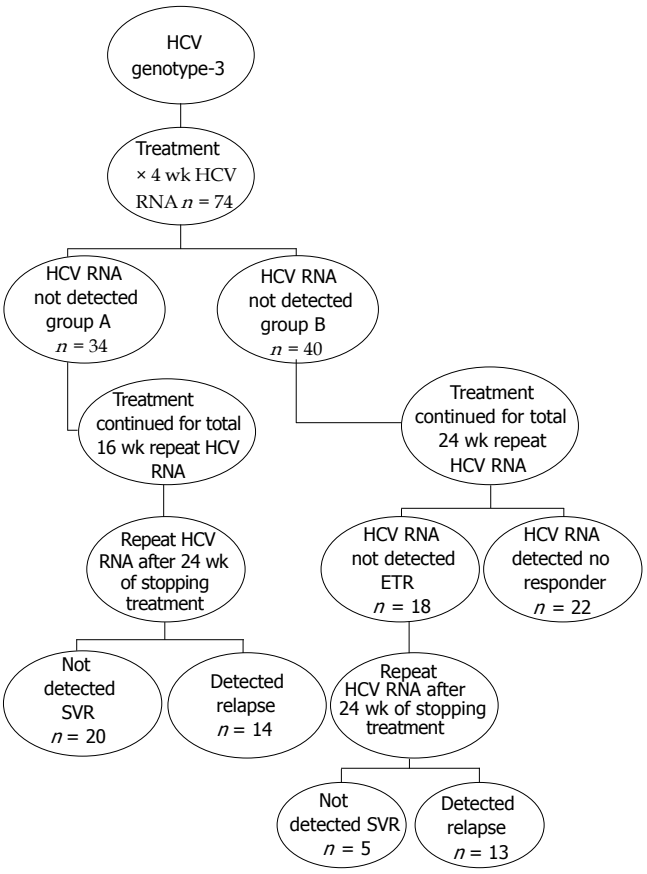
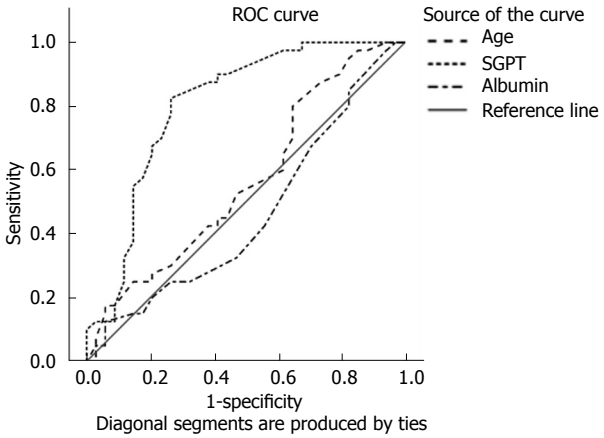


Figure 1 Treatment algorithm flowchart.



Area under the curve

Test result variable (s)	Area	Std. Error (a)	Asymptotic Sig. (b)	Asymptotic 95% CI	
				Upper bound	Lower bound
Age	0.566	0.068	0.332	0.433	0.698
ALT	0.802	0.054	0.000	0.696	0.909
Albumin	0.460	0.068	0.554	0.326	0.594

Figure 2 ROC Curve for age, ALT and Albumin for relapse.

Most of the studies with RVR were done in western countries which have a high prevalence of genotype-1. We report our results with SdIF in genotype-3 HCV; with about 46% achieving RVR and about 59% among them achieving SVR. A recent study by Yu *et al*[20] with PgIF reported RVR and SVR at 86% and 94% respectively. Another recent study by Yu *et al* reported RVR with PgIF in genotype-3 at 60%[17]. Cost effectiveness of the RVR based therapy with PgIF is established by recent reports not only in HCV infection alone but also in combined HCV/HIV infections[7,8]. In Pakistan the majority of genotype-3 patients are treated with SdIF due to economic reasons and Pakistan Society of Gastroenterology and GI Endoscopy also favours the use of SdIF in genotype-3[21]. Government is also providing only SdIF *via* a special Prime Minister's initiative for a viral hepatitis program, thus PgIF is out of reach for the majority of the patients.

The results of our study with SdIF are not comparable to that with PgIF in genotype-3 HCV on both RVR and SVR. In our study the SVR rates in patients who achieved RVR were about 59% and over all both groups combined SVR was 33% only which are quite low as compared to the recent reports of 86% and 90%[20]. Our study did show that SVR rates were higher in patients who attained RVR showing that RVR is an important landmark in management of HCV with SdIF too.

No data is available for prediction of RVR in patients undergoing treatment for HCV, although some data is available for SVR. One such study has been reported that at the age of 20 years; no cirrhosis/bridging fibrosis; ALT quotient = 7; body mass index 20 kg/m^2; viral load 40×10^6 IU/L was associated with a 97% probability of SVR[22].

In this study we tested the pretreatment levels of ALT, albumin and age as a prediction for RVR by plotting the ROC plots. Only ALT was found a significant marker as patients with high ALT were more likely to achieve RVR.

In conclusion, SVR rates were higher in patients who had RVR with SdIF and high pre treatment ALT values correlated to probability of having RVR.

COMMENTS

Background

Treatment of Hepatitis C virus (HCV) has varied response according to the genotype of the infecting virus. Ability to achieve Sustained Virological Response (SVR), which is the HCV polymerase chain reaction (PCR) negativity maintained six months after stopping treatment, is the real objective of the treatment. It is difficult to predict who will achieve Rapid virological response (SVR).

Research frontiers

Introduction of SVR is the clearance of HCV RNA within one month of treatment. It is being projected as a marker for SVR in patients treated with pegylated interferon. RVR has not been studied for SVR with standard interferon.

Innovations and breakthroughs

The study documents that in HCV genotype-3 patients treated with standard interferon SVR is related to the RVR. It was also shown that patients with initial high ALT are more likely to have RVR.

Applications

This will allow for better prediction of treatment results.

Terminology

SVR: Sustained virological response is defined as HCV RNA negativity six months after stopping the treatment. RVR: Rapid virological response is HCV RNA negativity after one month of treatment.

Peer review

Authors have shown that a short course of short acting interferon (16 wk) is efficacious in the patients who experience EVR. Perhaps, this approach would be cost-effective and useful in Pakistan.

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RAPID COMMUNICATION

Cost saving by reloading the multiband ligator in endoscopic esophageal variceal ligation: A proposal for developing countries

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Abstract

AIM: To assess the cost savings of reloading the multiband ligator in endoscopic esophageal variceal ligation (EVL) used on the same patient for subsequent sessions.

METHODS: This single centre retrospective descriptive study analysed patients undergoing variceal ligation at a tertiary care centre between 1st January, 2003 and 30th June, 2006. The multiband ligator was reloaded with six hemorrhoidal bands using hemorrhoidal ligator for the second and subsequent sessions. Analysis of cost saving was done for the number of follow-up sessions for the variceal eradication.

RESULTS: A total of 261 patients underwent at least one session of endoscopic esophageal variceal ligation between January 2003 and June 2006. Out of 261, 108 patients (males 67) agreed to follow the eradication program and underwent repeated sessions. A total of 304 sessions was performed with 2.81 sessions per patient on average. Thirty-two patients could not complete the programme. In 76 patients (70%), variceal obliteration was achieved. The ratio of the costs for the session with reloaded ligator *versus* a session with a new ligator was 1:2.37. Among the patients who completed esophageal varices eradication, cost saving with reloaded ligator was 58%.

CONCLUSION: EVL using reloaded multiband ligators for the follow-up sessions on patients undergoing variceal eradication is a cost saving procedure. Reloading the ligator thus is recommended especially for developing countries where most of the patients are not health insured.

Key words: Esophageal varices; Reloading; Multiband ligator; Eradication; Cost saving

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INTRODUCTION

Variceal hemorrhage is a major cause of death among patients with cirrhosis, carrying historically, a mortality rate of up to 50% before the advances in medicine[1,2]. Even with the advent of intensive care, vasoactive medications, and endoscopic therapies, the risk of death with variceal hemorrhage is still about 20% per episode[3,4].

Band ligation of esophageal varices is indicated as a primary prophylaxis for large varices and as a secondary prophylaxis for patients who have bled from varices[5]. It is the endoscopic procedure of choice to prevent recurrent variceal hemorrhage and eradicate varices which usually requires 3-4 sessions[6,7]. Endoscopic variceal ligation (EVL) is an expensive procedure, especially for patients from lower socioeconomic class in developing countries where health insurance and reimbursement systems are not as developed as in other countries. Most of the expenses are due to the high costs of the single polyband ligator use. Thus, reloading this ligator and re-using it for subsequent sessions on the same patient would substantially reduce costs, while also improving compliance to the eradication program as most of the patients are not covered by a health care scheme.

The aim of this study is to review the patients in the eradication program for esophageal varices and estimate the cost saving by using the reloaded band ligator to achieve this purpose.

MATERIALS AND METHODS

A retrospective analysis on 261 patients who had undergone EVL as primary or secondary prophylaxis between 1st January 2003 and 30th June 2006 was performed.

Saeed's Six Shooter Multi-Band Ligator (Cook Medical Inc, Bloomington, IN) was used for variceal ligation[8]. After each session all the accessories of the ligator were disinfected in glutaraldehyde solution (Cidex, Johnson & Johnson) by standard protocols. The band ligator was then reloaded with six hemorrhoidal bands for the 2nd and subsequent sessions on the same patient. We used hemorrhoidal band ligator for reloading barrel of the variceal ligator[9]. The procedure was approved by the Infection Control Committee of the hospital.

The procedures were performed by physicians experienced in the techniques of endoscopic ligation and sclerotherapy. Informed consent was obtained from the patients. Endoscopy was carried out under topical or pharyngeal anesthesia and sedation with intravenous midazolam if needed. Ligation was performed beginning at the most distal discernible extent of a variceal column and proceeding proximally. Subsequent endoscopic therapy sessions with EVL or combination therapy were performed at 14 to 21 d intervals until the varices were eradicated or reduced to grade one. Recurrent bleeding mandated unscheduled intervention.

Method for reloading

The plaited string or trigger cord of the multiple band ligator becomes separated into two threads near the barrel. Each thread has six beads at regular intervals starting from the tip of the thread. These threads are passed through the barrel of the multiple band ligator from its scope-end side and delivered from the transparent rim side. The banding apparatus is now loaded. The metal cone of the hemorrhoidal ligator is loaded with a band, and then fitted in to the cylinder of the hemorrhoidal ligator and the rubber band rolled from the cone to the cylinder. The cone is removed after charging the cylinder. The first tip (bead) of each thread is brought at the base of the transparent cap and held in position. The transparent rim of the barrel is slid into the cylinder and the handle of the hemorrhoidal ligator is closed to push off the band from the cylinder onto the barrel of the variceal ligator. The band is positioned to the base of the barrel's transparent portion above the first pair of beads. The next pair of beads is now brought above the first band, wrapping the portion of thread between the first and second bead on the barrel by repositioning second beads to 180 degrees. When the two beads are in position above the first band, the second band is applied. In this way all the bands are mounted on the barrel which is now ready for reuse.

RESULTS

A total of 261 patients underwent at least one session of EVL between January 2003 and June 2006. Patients undergoing sclerotherapy were not included in the study.

Out of 261, 108 patients agreed to follow the eradication program with reloaded band ligator and underwent a total of 304 sessions. Sixty-seven (62%) patients were males. They underwent 2.81 sessions on average. Twenty patients came only for one follow up session, while 12 patients underwent more than one follow-up session but did not complete esophageal varices eradication. Thus, a total of 76 (70%) patients participating in the program achieved eradication. These 76 patients completed esophageal varices eradication in 215 sessions (average 2.83). The reloaded ligator was used in a total of 139 follow-up sessions. The ratio of costs for the session with the reloaded ligator *versus* a first session with a new ligator was 1:2.37. Among the patients who completed the program and achieved eradication of esophageal varices, cost saving with reloading was 58% (Table 1).

Table 1 Cost savings after the first session in 76 patients who completed the eradication of varices. Cost is in US Dollars (1 US Dollar = 61 Pakistani Rupees)

	Reloaded band after first session	New six shooter used each time
Cost of EGD	91.8	91.8
Cost of bands/ligator	6.56	140.82
Cost of single follow up session	98.36	232.62
Cost of bands in 139 follow up session	911.47	19573.93
Total cost of 139 follow-up sessions	13672.13	32334.59
Average cost savings per patient	245.56	
Cost comparison	1	2.37
Overall cost saving	58%	
Cost saving in band ligators	95%	

Cost of EGD includes both the costs of the technical (i.e. equipment and facility costs) and professional fees.

The etiologies of esophageal varices among the patients in the eradication program included hepatitis C in 49 (64.5%), hepatitis B in 3 patients (3.9%), hepatitis B & D in 6 (7.9%), non-B non-C in 16 (21.1%), and alcoholic liver disease in 2 patients (2.6%).

DISCUSSION

Cirrhosis and complications of portal hypertension rank among the top 10 leading causes of death worldwide[10]. The prevalence of esophageal varices in patients with cirrhosis ranges from 12% to 90% and the average risk of bleeding from 14% to 78%, depending on the patient population studied[11]. Esophageal varices are the most common cause of significant gastrointestinal bleeding secondary to portal hypertension[12]. The acute mortality of variceal hemorrhage has been reported to be 15%-50% and the overall mortality within 1-4 years as high as 70%-80% in those with cirrhosis. Furthermore, once varices have bled, the risk of rebleeding is reported to be as high as 70%-80%.

Treatment of patients with esophageal varices includes the prevention of the initial bleeding episode (primary prophylaxis), the control of active hemorrhage, and the prevention of recurrent bleeding after a first episode

(secondary prophylaxis), for which several modalities have been used including endoscopic sclerotherapy and band ligation.

EVL is superior to sclerotherapy, and is considered to be the endoscopic treatment of choice for bleeding varices[8]. Placing a rubber band around the variceal vein induces venous obstruction followed by mucosal inflammation, necrosis, and obliteration of the variceal vein. The single-shot mechanism of the ligation device is inherently inefficient, and makes the procedure tedious. It also requires overtube placement, associated with discomfort and complications[13-15]. Multiple-band ligation devices make band ligation easier and more efficient, allowing the consecutive application of 5 to 10 bands without removing the endoscope.

Reuse of equipment will always be cheaper than using new equipment. The issue becomes important when patients have to pay for all medical costs themselves and are not covered by a health care plan. The main issue is safety of reusable equipment. There were no band ligator failures or other complications noted in our patients with reloaded equipment. Very occasionally an extra band slipped off while deploying. There were no infection issues in these patients. Reuse of 'disposable' medical equipments may be a source of infection for HBV, HCV, and HIV in less developed countries. We disinfected the disposable items of the ligator with glutaraldehyde according to the standard recommendations and closed in a sealed bag with a label of patient's identification details and stored in an allocated dry place in the endoscopy suite. On arrival of the patient, the bag was opened and the ligator was reloaded with aseptic precautions to be used on the same patient. It is not too difficult to reload the band ligator. The process takes about five minutes

Variceal eradication was achieved in 70% of the patients enrolled in our eradication program. A wide range of success rates in eradication of esophageal varices has been reported in several studies. In the study by Stiegmann *et al*[16], variceal obliteration occured in 27 patients of 64 (42%) while in the study by Lo *et al*[17] varices were eradicated in 74%. Cost savings of the whole procedure using reloaded band ligator were 58%. Cost saving of the ligators, if reloaded equipment was used, was 95%. The band ligator was virtually free as only the costs of the rubber bands was charged. Rest of the expenses was related to the endoscopy and recovery.

In conclusion, EVL using reloaded polyband ligators for the follow-up sessions on patients undergoing variceal eradication is a cost effective procedure and may be recommended for developing countries.

COMMENTS

Background

Band ligation of esophageal varices is indicated as a primary prophylaxis for large varices and as a secondary prophylaxis for patients who have bled from varices. It is the endoscopic procedure of choice to prevent recurrent variceal hemorrhage and to eradicate varices. It usually requires 3-4 sessions using multiband ligator and applying up to six bands each time. Endoscopic variceal ligation (EVL) is an expensive procedure, especially for patients from lower socioeconomic class in developing countries.

Research frontiers

Instead of using new multiband ligator for each session, reloading the ligator and using it for subsequent sessions on the same patient would substantially reduce the costs.

Related publications

Not much published work related to this aspect is available. We described the method of reloading of the variceal multiple band ligator using hemorrhoidal banding apparatus (letter). *J Pak Med Asso* (JPMA) 2000; 50: 285-286.

Innovations and breakthroughs

Cost saving of the whole procedure using reloaded band ligator was 58% of the cost had new ligator been used. The band ligator is virtually free as only the cost of the rubber bands is charged. Rest of the expenses is related to the endoscopy and recovery.

Applications

EVL using reloaded polyband ligators for the follow-up sessions on patients undergoing variceal eradication is a cost effective procedure and may be recommended for developing countries.

Terminology

Multiband ligator is a device used to ligate esophageal varices, allowing the consecutive application of six rubber bands without removing the endoscope.

Peer review

This article reports Pakistanis experience about EVL using reloaded multiband ligator. I have a great experience about this procedure in my department. It is indicated for the third world countries. This is a nice, simple, and short paper with a clear point.

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RAPID COMMUNICATION

Effect of thermal cutaneous stimulation on the gastric motor activity: Study of the mechanism of action

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Abstract

AIM: To investigate the mechanism of action of thermal cutaneous stimulation on the gastric motor inhibition.

METHODS: The gastric tone of 33 healthy volunteers (20 men, mean age 36.7 ± 8.4 years) was assessed by a barostat system consisting of a balloon-ended tube connected to a strain gauge and air-injection system. The tube was introduced into the stomach and the balloon was inflated with 300 mL of air. The skin temperature was elevated in increments of 3℃ up to 49℃ and the gastric tone was simultaneously assessed by recording the balloon volume variations expressed as the percentage change from the baseline volume. The test was repeated after separate anesthetization of the skin and stomach with lidocaine and after using normal saline instead of lidocaine.

RESULTS: Thermal cutaneous stimulation resulted in a significant decrease of gastric tone 61.2% ± 10.3% of the mean baseline volume. Mean latency was 25.6 ± 1.2 ms. After 20 min of individual anesthetization of the skin and stomach, thermal cutaneous stimulation produced no significant change in gastric tone.

CONCLUSION: Decrease in the gastric tone in response to thermal cutaneous stimulation suggests a reflex relationship which was absent on individual anesthetization of the 2 possible arms of the reflex arc: the skin and the stomach. We call this relationship the "cutaneo-gastric inhibitory reflex". This reflex may have the potential to serve as an investigative tool in the diagnosis of gastric motor disorders, provided further studies are performed in this respect.

INTRODUCTION

External stimuli have been shown to affect gastric motility. Centrally acting stressful stimuli produce gastrointestinal motility changes in rats[1-6], dogs[7,8], and humans[9-11]. These actions seem to be mediated through humoral pathways[12-14]. Thus α- and β-adrenergic blockers are claimed to abolish the inhibition of gastric motility induced by cold pain in humans[15]. Other investigators suggest that other humoral factors, such as acoustic stress, may be involved in the mediation of the gastrointestinal motor disturbances[16-19]. Gastric ulcer and acute pancreatitis may also be related to stress[11,20].

It has been shown that various types of stressors cause a release of the corticotrophin-releasing factor and that intra-cerebroventricular administration of this factor mimics the motor, metabolic, and hemodynamic responses to such stimuli in animals[21-24]. However, in anesthetized rodents, an increased gastric motility was observed during restraint stress[25,26], whereas pinching of the skin was accompanied by gastric motor inhibition[20].

Although skin pinching or stressful cutaneous stimuli have been demonstrated to be associated with gastric motor inhibition[27,28], the mechanisms involved in this action have not been elucidated in the literature. Therefore, hypothesizing that skin stimulation induces its effect on the gastric motor activity through a reflex action, we conducted the current study.

MATERIALS AND METHODS

Subjects

Thirty-three subjects [20 men and 13 women; mean age 36.7 ± 8.4 (range 26-45) years] were enrolled in this

study after they had given an informed consent. The results of physical examination including neurological assessment were normal. Laboratory work up including blood count, renal and hepatic function tests, as well as electrocardiography were normal. The study was approved by the Faculty of Medicine Review Board and Ethics Committee of Cairo University.

Methods

Thermal cutaneous stimulation (TCS) was performed by means of a thermal pad applied to the skin, and the gastric motor activity was recorded with a barostat. A 6F polyvinyl gastric tube, with multiple side holes 4 to 6 cm from its distal end, was used. A thin compliant polyethylene balloon (London Rubber Industries Ltd, London, UK) was fastened to the distal part of the tube that contained the side holes. The tube had a metallic clip applied to its distal end for fluoroscopic control. It was connected to a strain gauge and a computer-controlled air-injection system (G&J Electronics Inc, Toronto, Ontario). This barostat system keeps the pressure within the balloon constant. Thus, when the gastric tone increases, the air in the bag is withdrawn, and when the tone diminishes, the air rushes into the balloon; hence the pressure in the balloon is kept constant at all times. Using this technique, the gastric tone could be assessed by recording the balloon volume variations, expressed as the percentage change from the baseline volume.

The tube was introduced into the stomach through the nose**.** The tests were performed 20 min later so that the stomach would have adapted to the inserted catheter**.** The balloon was then inflated with 300 mL of air. Thermal stimulation of the skin was induced by a thermal pad applied to the skin of the upper arm and connected to a thermostat.

The skin temperature was recorded at rest. The pad temperature was then elevated in increments of 3℃ above the resting skin temperature up to 49℃ or the highest tolerable temperature. Throughout the period of successive skin temperature elevation, the gastric wall tone was simultaneously assessed by measuring the variations in the balloon volume, expressed as the percentage change from the baseline volume. We calculated the latency which is the period between the start of thermal skin stimulation and the beginning of the gastric tone response.

To define whether the effect of thermal cutaneous stimulation (TCS) on the stomach was a direct or a reflex action, the following test was performed.

Cutaneous and gastric anesthetization

The aforementioned test was repeated after individual anesthetization of the skin and stomach. The skin area, over which the thermal pad was applied, was anesthetized by injection of 3 mL of 2% lidocaine mixed with 3 mL of normal saline; the injection was performed at multiple points in the skin under the pad. The gastric tone response to TCS, as aforementioned, was recorded after 20 min and 3 h later when the anesthetic effect had waned. The stomach was then anesthetized by endoscopic injection of 30 mL of 2% lidocaine in 70 mL of normal saline. The injection was performed at multiple points in the stomach wall. The gastric tone response to TCS was then registered after 20 min and 3 h later. The aforementioned tests were repeated using normal saline instead of lidocaine.

Table 1 Change in the gastric tone in response to the different degrees of thermal cutaneous stimulation (mean ± SD)

Skin temperature (℃)	Basal tone (% of baseline volume)	
	Mean	Range
37 (basal)	0	0
40	48.2 ± 6.4	40-56
43	57.3 ± 5.1	52-63
46	69.7 ± 3.3	66-74
49	78.6 ± 4.1	76-83

RESULTS

The study was completed without any adverse side effects during or after the tests. During TCS, all of the subjects showed a significant decrease in the gastric tone which varied from 40% to 83% (mean 61.2% ± 10.3%) of the baseline volume according to the degree of TCS (Figure 1, Table 1). There was a progressive decrease in the gastric tone with increasing TCS (Figure 1, Table 1). Gastric tone decline was greater in men than in women but the difference was not significant ($P > 0.05$). Also, there was no significant difference in the gastric tone decrease between the younger and older subjects. The latency varied from 20.6-28.8 ms (mean 25.6 ± 1.29). It decreased with increasing TCS. There was no significant difference in the latency when we compared men to women or younger to older subjects.

Effect of TCS on the gastric tone after individual cutaneous and gastric anesthetization

TCS performed 20 min after individual anesthetization of the skin or stomach produced no significant changes in the gastric tone (Figure 2). Three hours later, when the anesthetic effect had waned, the TCS caused a decrease in gastric tone similar to that before anesthetization ($P > 0.05$). When the above tests were repeated using saline instead of lidocaine, the gastric tone response was similar to that before saline application ($P > 0.05$).

Upon repetition of the aforementioned tests in the same subject, similar results were obtained with no significant differences.

DISCUSSION

It is established that centrally acting stressful stimuli induce changes in the gastrointestinal motility[1-11]. These changes are suggested to occur through humoral pathways as supported by the release of β-endorphin and catecholamines into the peripheral circulation during stress[12-15]. In agreement with such hypothesis, naloxone or a combination of α- and β-adrenergic blockers abolish gastric motility inhibition induced by cold pain. Yet, this hypothesis can be ruled out because the aforementioned drugs have no effect on the migrating duodenal activity

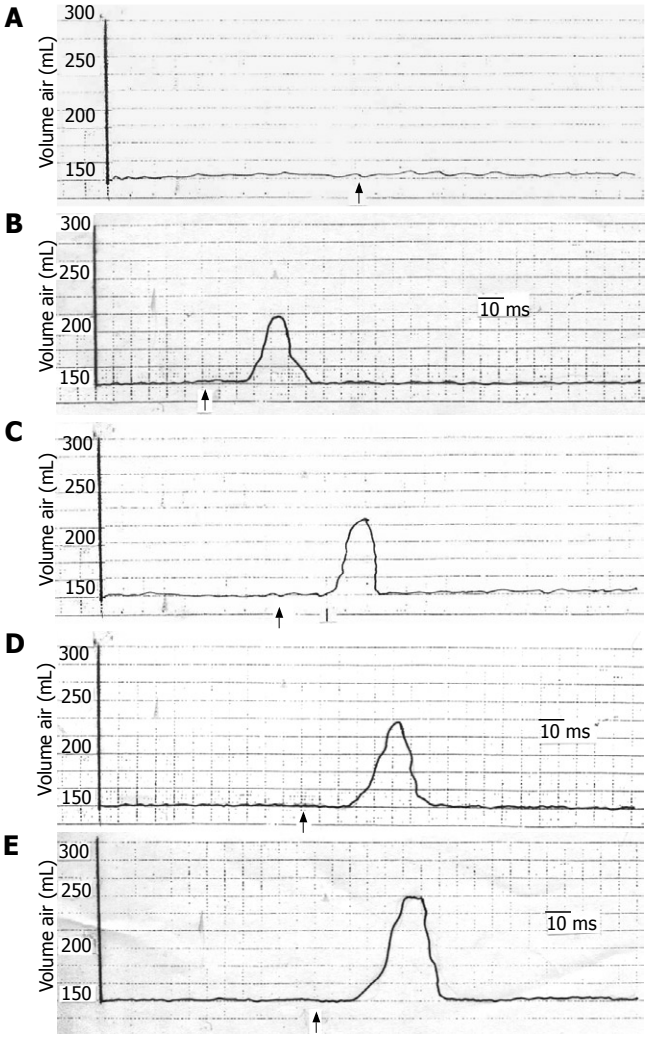

Figure 1 Decrease in the gastric tone in response to thermal cutaneous stimulation (arrow). (**A**) 37℃ (basal); (**B**) 40℃; (**C**) 43℃; (**D**) 46℃; and (**E**) 49℃.

induced by labyrinthine stimulation in humans[24-28] or on acoustic stress-induced inhibition of gastric motility in dogs[27]. This suggests that other factors may be involved in the mediation of gastrointestinal motor disturbances induced by centrally acting stimuli.

The current study demonstrated that TCS affected inhibition of gastric motor activity which progressively increased on incremental enhancement of TCS. This effect was abolished on anesthetization of either the stimulated cutaneous area or the stomach. The inhibited gastric motor activity presumably denotes gastric wall relaxation and gastric dilatation. It seems that the stomach dilates on stress to avoid gastric stimulation that might result in vomiting.

The current findings led to the assumption that the inhibited gastric motor activity in response to cutaneous stressful condition is mediated through a reflex pathway. This hypothesis is evidenced by the findings that, with individual anesthetization of the suggested 2 arms of the reflex arc, i.e. the skin and the stomach, the gastric response was absent. Saline on the other hand did not give rise to such effect. The response returned after the anesthetic condition had worn off. Furthermore, the reproducibility of the effect points to the constancy

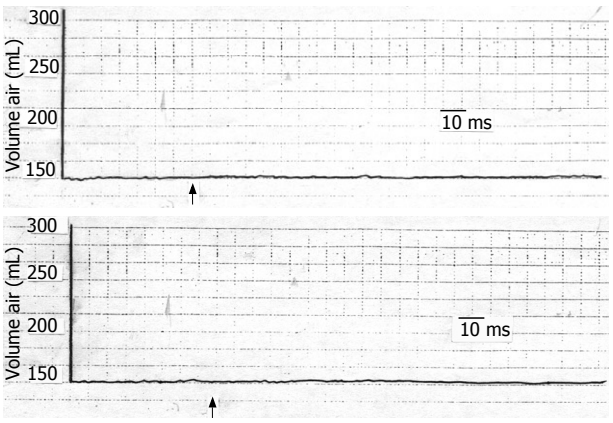

Figure 2 Response of the gastric tone to thermal cutaneous stimulation (arrow) at 46℃ 20 min after separate anesthetization of the skin (**A**) and the stomach (**B**).

of the results. We call the suggested reflex response of the stomach to cutaneous stimulation, the "cutaneo-gastric inhibitory reflex (CGIR)". It may be argued that this effect could be humoral as already mentioned by investigators[10-18]. However, if the effects of cutaneous stimulation on the stomach were humoral, it would not vanish with either gastric or cutaneous anesthetization as has been shown in the current findings. Meanwhile, the effect of centrally acting stressful stimuli on the stomach[1-6] cannot be ignored, albeit that this role alone does not seem to explain the non-response of the stomach to stimulation of the anesthetized skin.

It seems that TCS activates the cutaneous nerve endings which send impulses along the afferent fibers to the spinal cord. Impulses from the spinal cord are in turn transmitted along efferent fibers to the stomach, inhibiting its motor activity.

The point that needs to be discussed is: what could be the possible clinical significance of the CGIR? It is suggested that the CGIR might be of diagnostic significance in gastric motility disorders. Diminished gastric tone response to TCS would indicate a defect in the reflex pathway, such as gastric musculature or nerve damage resulting from a disease of the peripheral nerves, spinal nerve roots or spinal cord or from a central lesion. Significant prolongation of the latency of the CGIR on the other hand may indicate a disorder of the reflex arc. We believe that the CGIR may be incorporated as an investigative tool in the study of patients with gastric disorders after it has been further studied in various pathologic gastric lesions. The reflex assesses the integrity of the gastric motor activity.

In conclusion, TCS results in decrease of the gastric motor activity which apparently leads to gastric wall relaxation. The decrease in gastric tone upon TCS postulates a reflex relationship which was absent on individual anesthetization of the assumed two arms of the reflex are: the skin and the stomach. We call this relationship the CGIR. This reflex may prove to be of diagnostic significance in gastric motor disorders and have the potential to serve as an investigative tool, provided further studies are performed to validate the current results.

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COMMENTS

Background

External stimuli have been shown to affect gastric motility. Centrally acting stressful stimuli produce gastrointestinal motility changes in rats, dogs, and humans. These actions seem to be mediated through humoral pathways. Thus α- and β-adrenergic blockers are claimed to abolish the inhibition of gastric motility induced by cold pain in humans. Other investigators suggest that other humoral factors such as acoustic stress may be involved in the mediation of the gastrointestinal motor disturbances. We hypothesized that skin stimulation induces its effect on the gastric motor activity through a reflex action. This hypothesis was investigated in the current study.

Research frontiers

It is established that centrally acting stressful stimuli induce changes in the gastrointestinal motility. Other factors may be involved in the mediation of gastrointestinal motor disturbances induced by centrally acting stimuli.

Innovations and breakthroughs

The point that needs to be discussed is: what could be the possible clinical significance of the cutaneo-gastric inhibitory reflex (CGIR)? It is suggested that the CGIR might be of diagnostic significance in gastric motile disorders. Diminished gastric tone response to thermal cutaneous stimulation (TCS) would indicate a defect in the reflex pathway, such as gastric musculature or nerve damage resulting from a disease of the peripheral nerves, spinal nerve roots or spinal cord or from a central lesion. We believe that the CGIR may be incorporated as an investigative tool in the study of patients with gastric disorders after it has been further studied in various pathologic gastric lesions. The reflex assesses the integrity of the gastric motor activity.

Peer review

The authors had asked a simple question, namely whether gastric tone responds to cutaneous stimulation with heat. The answer is straight forward: Heat application leads to gastric relaxation and this effect can be abolished by intracutaneous and intragastric injections of lidocaine.

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RAPID COMMUNICATION

DNMT3B 579 G>T promoter polymorphism and risk of esophagus carcinoma in Chinese

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Abstract

AIM: To investigate the relationship between 579 G>T polymorphisms in the DNMT3B gene, which is involved in de novo methylation and associated with the risk of esophagus cancer (EC) in Chinese.

METHODS: DNMT3B 579 G>T genotypes were determined by PCR-RFLP in 194 EC patients and 210 healthy controls matched for age and sex, who did not receive radiotherapy or chemotherapy for newly diagnosed and histopathologically confirmed EC.

RESULTS: In control subjects, the frequency of T/T and G/T genotypes, and T and G alleles was 80.5%, 19.0%, 90.0% and 10.0%, respectively. The distribution of genotypes and allelotypes in the EC patients was not significantly different from that in the controls. When stratified by sex and age, there was still no significant association between the risks of EC and GT and GG genotypes. This study also showed a distinct difference in the distribution of DNMT3B and single nucleotide polymorphism (SNP) between Chinese and Koreans.

CONCLUSION: DNMT3B 579 G>T polymorphism may not be a stratification marker to predict the susceptibility to EC, at least in Chinese. DNMT3B promoter SNP is diverse in ethnic populations.

Key words: Esophagus cancer; DNMT3B; Methylation; Polymorphism

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INTRODUCTION

Esophagus carcinoma (EC) is one of the most common malignancies and the main cause of cancer-related death in the world. Because symptoms typically remain absent until late in the course of disease, most cancers are detected at an advanced stage when prognosis is poor. Therefore, it is important to investigate the genetic and epigenetic variation in susceptibility to esophagus carcinogenesis and identify the markers that will facilitate identification of individuals at risk of esophagus carcinogenesis.

DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5' position of a cytosine in a CpG dinucleotide. A number of studies suggested that aberrant DNA cytosine methylation may play an important role in carcinogenesis[1-5]. DNMT3A and DNMT3B are required for the establishment and maintenance of genomic methylation patterns and proper murine development[6-9]. Both genes are up-regulated to different degrees in some malignancies, including colon cancer and EC[10-14]. Recently, several candidate single nucleotide polymorphisms (SNPs) in the DNMT3B gene have been deposited in public databases. Although the functional effects of these polymorphisms have not been elucidated, some studies showed that some of these variants may influence the DNMT3B activity on DNA methylation,

thereby modulating the susceptibility to lung cancer, breast cancer and gastric cardiac adenocarcinoma[15-17]. The DNMT3B gene contains a single G>T SNP in the transcription start site of the promoter region (-579 bp from exon 1B), and this probably affects gene function[18]. Some studies suggested that DNMT3B -579 G>T may modify susceptibility to tumors. Although conflicting results have been reported in different tumor types, the heterozygous genotypes have a significantly reduced risk of developing lung and colon cancer[19-21]. However, no report on the association between this allele and the development of EC is available. This study was to investigate the association between this polymorphism and EC in Chinese.

MATERIALS AND METHODS

Study population

This case-control study included 194 EC patients and 210 healthy controls. EC was histopathologically confirmed in the 194 patients during surgery at the Zhongda Hospital of Southeast University and Tumor Hospital, Nanjing, China. The control subjects were selected from cancer-free subjects who visited the same hospital for a regular physical examination and volunteered to participate in the epidemiology survey during the same period. We defined a healthy subject as a person free of disease (including no history of cancer) at health check-up. The controls were matched for age and sex with the patients (Table 1). All patients and controls were ethnically Chinese and resided in Jiangsu Province or in its surrounding regions.

DNA extraction

Five milliliters of venous blood was drawn from each subject into vacuum tubes containing EDTA and stored at 4℃. Genomic DNA was extracted within one week after sampling by proteinase K digestion and salted out as previously described[19].

DNMT 3B genotyping

Transition from G to T of the DNMT3B SNP creates a *PvuII* restriction site, which can be exploited for genotyping by PCR and subsequent restriction fragment length polymorphism (RFLP) analysis. PCR was performed in a volume of 25 μL containing 100 ng of DNA template, 10 × PCR master mix (Promega, USA), and 10 pmol/L each of sense primer (5'-GAGGTCTCATTATGCCTAGG-3') and antisense primer (5'-GGGAGCTCACCTTCTAGAAA -3'). For PCR amplification, an initial denaturation at 94℃ for 5 min was followed by 30 cycles at 94℃ for 30 s, at 57℃ for 30 s, at 72℃ for 30 s, and a final extension at 72℃ for 7 min. The PCR products were digested overnight with 5 units of *PvuII* (New England Biolabs, Beverly, Mass.) at 37℃ and separated on 2% agarose gels. RFLP bands were visualized under UV light with ethidium bromide staining. The DNMT3B T/T genotype was expected to show two DNA bands at the positions of 132 bp and 93 bp, whereas the G/G genotype was expected to show a single band (225 bp), and the heterozygote was expected to have three bands (225 bp, 132 bp, 93 bp). For quality control, genotyping analysis was performed blindly with respect to case/control status and repeated twice for all subjects.

Table 1 Distribution of selected variables in esophagus cancer patients and control subjects *n* (%)

Variables	Patients (*n* = 194)	Control (*n* = 210)	*P*
Age (yr)			> 0.05
< 40	1 (0.5)	2 (0.9)	
40-60	117 (60.3)	110 (52.4)	
61-80	76 (39.2)	98 (46.7)	
Sex			> 0.05
Male	150 (77.3)	146 (69.5)	
Female	44 (22.7)	64 (30.5)	

DNA sequencing analysis

To confirm the genotyping results, selected PCR-amplified DNA samples were examined by DNA sequencing. The PCR fragments were recovered from agarose gel followed by purification with a DNA clean-up kit (Wizard SV Gel and PCR Clean-up System, Promega). DNA sequences of the PCR products were determined using the PCR sense primer with an Applied Biosystems model 377 sequencer (PE Applied Biosystems, Warrington, UK). The results of genotyping were 100% concordant.

Statistical analysis

Patients and controls were compared using Student's *t*-test for continuous variables and chi square (χ^2) test for categorical variables. Hardy-Weinberg equilibrium was tested with a goodness-of-fit χ^2 test with one degree of freedom to compare the observed genotype frequencies with the expected genotype frequencies among the subjects. Comparison of the DNMT3B genotype and allelotype distribution in the study groups was performed by means of two-sided contingency tables using χ^2 test or Fischer's exact test. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model and adjusted for age and gender accordingly. $P < 0.05$ was considered statistically significant.

RESULTS

The demographics of the cases and controls enrolled in this study are shown in Table 1. There were no significant differences in the mean age and sex distribution between cases and controls, suggesting that the matching based on these two variables was adequate. There was no evidence of a deviation from Hardy-Weinberg equilibrium among the cases or controls. The mean age of the patients and controls was 59.6 years (± 10.2 years; range, 34-80 years) and 59.6 years (± 10.2 years; range, 34-80 years), respectively.

All the patients and controls were successfully genotyped for the DNMT3B polymorphism (Figure 1). The genotyping by PCR-RFLP analysis was completely confirmed by DNA sequencing analysis, and the results of PCR-RFLP genotyping and sequencing analysis were also 100% concordant (Figure 2). The distribution of DNMT3B 579 G>T polymorphism was in Hardy-Weinberg equilibrium. The frequency of G allele in control subjects (0.10) was different from that in the previous

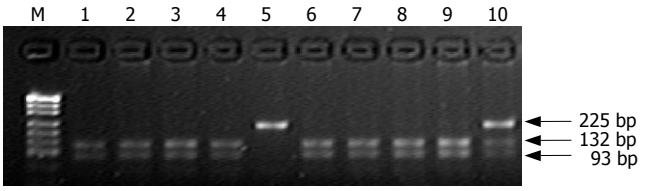

Figure 1 PCR-based restriction fragment length polymorphism genotyping of DNMT3B 579 G>T. Lanes 1-4, 6-9: TT variants; lane 5: GG wild type; lane 10: GT heterozygote.

Table 2 DNMT3B genotype and allele frequency in Chinese and Koreans

	TT	GT	GG	G allele frequency (%)
Chinese	169 (80.5)	40 (19.0)	1 (0.5)	10.0
Korean	153 (61.7)	91 (36.7)	4 (1.6)	20[b]

[b]$P < 0.01$ *vs* Chinese.

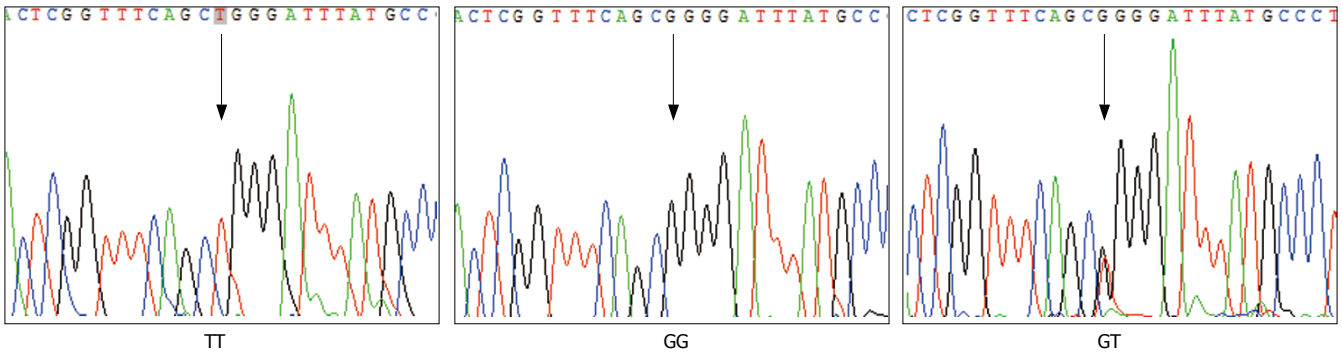

Figure 2 Sequencing results for each of the PCR products from different genotypes. The SNP sites are indicated by the arrowhead. The results were completely matched to the corresponding results derived from PCR-RFLP genotyping.

study among Koreans (0.20)[21]. The distributions of DNMT3B 579 G>T genotypes in Chinese and Koreans are shown in Table 2.

The distributions of DNMT3B 579 G>T genotypes in controls and patients are shown in Table 3. The genotype distributions of both polymorphisms in the controls were in Hardy-Weinberg equilibrium. No significant deviation was observed in the genotype distributions of both polymorphisms between overall esophagus cancer patients and controls. Then we stratified the results by sex and age, patients and controls were found to be slightly different with respect to the genotype distribution (Table 4). Combined GG and GT genotypes were found to have a little higher OR in male esophagus cancer patients and the group under the age of 59 years (males: OR 1.35 ; 95% CI, 0.76-2.39; under 59: OR 1.47 ; 95% CI, 0.74-2.90). However, combined GG and GT genotypes showed no significant association between DNMT3B 579 G>T polymorphism and the risk of esophagus cancer.

Table 3 DNMT3B genotype and allele frequency in patients and control subjects and their association with esophagus cancer *n* (%)

	Case patients (*n* = 194)	Control subjects (*n* = 210)	OR (95% CI)
DNMT3B 579 G>T			
TT	151 (77.8)	169 (80.5)	
GT	43 (22.2)	40 (19.0)	1.17 (0.73-1.90)
GG	0 (0)	1 (0.50)	
G allele (%)	11.1	10.0	

Table 4 Stratification analysis of DNMT3B 579 G>T genotype frequencies in esophagus cancer patients and controls, adjusted OR (95% CI)

Variable	TT genotype Case/control	GT + GG genotype Case/control	Odds ratios of GT + GG genotype
Age			
< 60	81/89	24/18	1.47 (0.74-2.90)
⩾60	70/80	19/23	0.94 (0.48-1.88)
Sex			
Male	116/120	34/26	1.35 (0.76-2.39)
Female	35/49	9/15	0.84 (0.33-2.14)

DISCUSSION

Single nucleotide polymorphism (SNP) is the most common form of human genetic variation, and may contribute to an individual's susceptibility to cancer. Studies suggested that some variants in the promoter region of genes may affect either the expression or activity levels of enzymes[1,18,22] and therefore may be mechanistically associated with cancer risk. It has been recently shown that SNP of the DNMT3B promoter 579 G>T (from exon 1B transcription start site) decreases the susceptibility of an individual to lung and colon cancer[20,21], suggesting that DNMT3B promoter 579 G>T polymorphism can be used as a risk factor for cancer to evaluate the population susceptible to tumors. However, it was also reported that there is no association between polymorphism of 579 G>T and head and neck squamous cell carcinoma[23]. However, to the best of our knowledge, the relative significance of SNP in the genetic susceptibility to esophagus cancer has not yet been disclosed. In the

current study, we investigated the influence of DNMT3B polymorphisms on the risk of esophagus cancer in a hospital-based case-control study.

This is the first study of DNMT3B polymorphism in esophagus cancer. We investigated the influence of 579 G>T polymorphism in the DNMT3B gene on the risk of esophagus cancer. Individuals carrying G allele in the DNMT3B gene were found to have a nearly consistent risk of EC compared with those carrying T allele. Then we stratified the results by sex and age, patients and controls. Combined GG and GT genotypes showed no significant association between DNMT3B 579 G>T polymorphism and risk of esophagus cancer, suggesting that 579 G>T polymorphism in the DNMT3B gene cannot be used as a marker of genetic susceptibility to esophagus cancer even in young individuals. Our study showed that DNMT3B polymorphism was not associated with the risk of esophagus carcinoma, at least in the study population, although other studies reported a decreased risk of lung and colon cancer in those harboring G allele. Since the different variants of DNMT3B may alter catalytic activity and are expressed in a tissue specific manner[24-27] and the repression of DNMT3B activity does not result in re-expression of all hypermethylated tumor suppressor genes in some cell systems[28-31], it is therefore important to explore the complex interplay of DNMTs in different tumor types.

In this study, a distinct difference was found in the distribution of DNMT3B SNP between Chinese and Koreans. However, few G/G genotypes were found in both populations. Additionally, the frequency of G/T genotype in Chinese was lower than that in Koreans. The great diversity in DNMT3B SNP distribution in different ethnic populations remains unknown.

In conclusion, the DNMT3B gene may not be involved in the development of esophagus cancer. Further studies with a larger sample are required to confirm our findings, to understand the role of DNMT3B polymorphisms in determining the risk of esophagus cancer, and to clarify the association of DNMT3B polymorphism with esophagus cancer in different ethnic populations.

COMMENTS

Background

DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5' position of a cytosine in CpG dinucleotide. DNMT3A and DNMT3B are required for the establishment and maintenance of genomic methylation patterns. Single nucleotide polymorphisms (SNPs) in the DNMT3B gene may influence DNMT3B activity on DNA methylation, thereby modulating the susceptibility to some cancer.

Research frontiers

Some variants in the promoter region of genes may affect either the expression or activity levels of enzymes and therefore may be mechanistically associated with cancer risk. It has been recently reported that SNP of the DNMT3B promoter 579 G>T (from exon 1B transcription start site) decreases the susceptibility of an individual to lung and colon cancer. However, it was also reported that there is no association between polymorphism of 579 G>T and head and neck squamous cell carcinoma. The relative significance of SNP in genetic susceptibility of an individual to cancer is diverse in different populations.

Innovations and breakthroughs

It is important to investigate the genetic and epigenetic variation in susceptibility to esophagus carcinogenesis and identify markers that will facilitate identification of individuals at risk of esophagus carcinogenesis. Although no significant association was found between DNMT3B 579 G>T polymorphism and risk of esophagus cancer, this is the first study of DNMT3B polymorphism in esophagus cancer. This study also showed the significance of great diversity in DNMT3B SNP distribution in different ethnic populations and their susceptibility to cancer.

Applications

A distinct difference was found in the distribution of DNMT3B SNP between Chinese and Koreans in this study. The significance of great diversity in DNMT3B SNP distribution in different ethnic populations remains unknown. These results suggest that the DNMT3B gene may not be involved in the development of esophagus cancer. Future studies of other DNMT3B sequence variants and their biologic function are needed to understand the role of DNMT3B polymorphisms in determining the risk of esophagus cancer.

Peer review

The association between DNMT3B 579 G>T polymorphism and the risk of esophagus cancer in Chinese was studied. The results show that the DNMT3B 579 G>T polymorphism was not associated with the risk of esophagus carcinoma, at least in the study population. This study also showed the significance of great diversity in DNMT3B SNP distribution in different ethnic populations and their susceptibility to cancer.

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RAPID COMMUNICATION

Anti-sense oligonucleotide labeled with technetium-99m using hydrazinonictinamide derivative and N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycline: A comparison of radiochemical behaviors and biological properties

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Abstract

AIM: To explore and compare the radiochemical behavior and biological property of anti-sense oligonucleotide (ASON) labeled with technetium-99m using N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycl ine (NHS-MAG_3) and hydrazinonictinamide derivative (HYNIC).

METHODS: After HYNIC and NHS-MAG_3 were synthesized, ASON was labeled with technetium-99m using HYNIC and NHS-MAG_3 as a bifunctional chelator. The *in vivo* and *in vitro* stability, binding rates of labeled compounds to serum albumen, biodistribution of ^{99m}Tc-MAG_3-ASON and ^{99m}Tc-HYNIC-ASON in BALB/C mouse and its HT29 tumor cellular uptake were compared.

RESULTS: The labeling efficiency and stability of ^{99m}Tc-MAG_3-ASON were significantly higher than those of ^{99m}Tc-HYNIC-ASON (P = 0.02, and P = 0.03, respectively). ^{99m}Tc-MAG_3-ASON had a significantly lower rate of binding to serum albumen than ^{99m}Tc-HYNIC-ASON (P < 0.05). In contrast to ^{99m}Tc-HYNIC-ASON, the biodistribution of ^{99m}Tc-MAG_3-ASON was significantly lower in blood, heart, liver and stomach (P < 0.05), slightly lower in intestines and spleen (P > 0.05) and significantly higher in lung and kidney (P < 0.05). The HT29 tumor cellular uptake rate of ^{99m}Tc-MAG_3-ASON was significantly higher than that of ^{99m}Tc-HYNIC-ASON (P < 0.05).

CONCLUSION: ^{99m}Tc-MAG_3-ASON shows superior radiochemical behaviors and biological properties than ^{99m}Tc-HYNIC-ASON. ^{99m}Tc-MAG_3-ASON is a potential radiopharmaceutical agent for *in vivo* application.

Key words: Anti-sense oligonucleotide; Radiolabeling; Technetium-99m; N-hydroxysuccinimidyl S-acetylmercapt oacetyltriglycline; Hydrazinonictionamide derivative

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INTRODUCTION

Different drugs can be used in anti-sense therapy, among which synthetic anti-sense oligonucleotide (ASON) is used to bind to deoxyribonucleic acid (DNA) translation or transcription in a sequence-specific manner and interfere with the expression of oncogene. However, it is still difficult for ASON to target tumor cells and transport across cell membrane. Besides, because of multi-gene expressions in tumor cells, inhibition of any single target gene is not sufficient to inhibit tumor growth. Radiolabeled ASON targeting specific oncogenes can overcome these problems by direct inhibition of anti-sense and radiation damage. The curative effect of radionuclide anti-sense therapy is closely related to the labeling efficacy of ASON and the characteristics of labeled compounds. In contrast to ^{188}Re, ^{186}Re, ^{90}Y[1-3], hydrazinonictinamide derivative (HYNIC) and N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycline (NHS-MAG_3), as a bifunctional chelator, have been known to help label

ASON[4-6] with ^{99m}Tc. However, few reports are available on the comparison of both chelators. This study was to compare the radiochemical behaviors and biological properties of ASON labeled with technetium-99m using NHS-MAG_3 and HYNIC.

MATERIALS AND METHODS

Materials

BALB/c nude mice at the age of 6-8 wk, weighing 17-22 g, were obtained from West China Experimental Animal Center. Human colon carcinoma HT29 cell line was obtained from the Laboratory of West China Hospital. HT29 cells were incubated in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin. Fifteen-mer phosphorothioate ASON (5'-NH_2-FACGTTGAGGGGCAT-3', F is adenosine sulfurised), which is complementary to the translation start site of c-myc mrRNA, was purchased from GiBcoBRL (USA). $^{99m}TcO_4^-$ (37 TBq/L) was obtained from Chengdu Gaotong Isotope Corporation (Chengdu, China). Sephadex G25 was from Pharmacia Fine Chemicals A.B (Uppsala, Sweden). C_{18} Sep-Pak reversed-phase column was a product from Waters Company (Milford, USA). CRC-15R dose calibrator was from Capintec Company (Ramsey, New Jersey, USA). FH463A automatic scaler was supplied by Beijing Nuclear Instrument Company (Beijing, China). Unity Inova-400 nuclear magnetic resonator was from Varian Company (USA). UV-2100 spectrophotometer was from Beckman Company (Cotati, California, USA). Frozen desiccator was from Marathon Electric Company (New York, USA). CO_2 incubator was from Sanyo Company (Japan). Centrifuge was from Beckman Company (Cotati, California, USA).

Synthesis of HYNIC and NHS-MAG_3

HYNIC was synthesized as previously described[7] (Figure 1). The end product was purified by recrystallization in isopropyl alcohol and the yield was 75%. The synthesis process of NHS-MAG_3 has been described elsewhere[6] (Figure 2). The melting temperature of the end product was 140℃-155℃, the yield was 80%. The content was 2.38 ppm (S, 3H, $SCOCH_3$), 2.80 ppm (S, 4H, succinimidyl), 3.68-3.80 ppm (M, 8H, $COCH_2$) and 8.20-8.38 ppm (M, 3H, NHCO), respectively, by nuclear magnetic resonance spectroscopy.

^{99m}Tc labeling ASON via HYNIC

ASON (2 mg/mL) buffer was dissolved in 2 mol/L NaCl, 0.5 mol/L $NaHCO_3$ and 2 mmol/L ethylenediamine tetraacetic acid (EDTA), and HYNIC (10 mg/mL) was dissolved in dimethylformamide (DMF). In 45℃ water bath, 31 μL HYNIC and 2 mmol/L EDTA were gradually dropped into a 25 μL ASON solution at the molar ratio of 25:1. The reaction system was filtered through a Sep-Pak C18 reversed-phase column (10 mm × 5 mm) in 60% methanol to remove HYNIC not binding to ASON. HYNIC-ASON was collected with a UV-2100 spectrophotometer and frosted to dry powder for storage. HYNIC-ASON dry powder was labeled on d 15, 30 or 60, respectively. Ten μg HYNIC-ASON powder was dissolved in a 0.5 mL tricine solution (70 mg/mL). $SnCl_2 \cdot 2H_2O$ solution (1 mg/mL) was dissolved in 0.1 mol/L HCl at room temperature. HYNIC-ASON solution, 25 μL $SnCl_2 \cdot 2H_2O$ solution and 0.2 mL $^{99m}TcO_4^-$ containing a radioactivity of 370, 740 or 1480 MBq were mixed uniformly. After stored for 30 min at room temperature, the mixture was eluted and purified through a Sep-Pak C18 reversed-phase column (10 mm × 5 mm) in 60% methanol, and ^{99m}Tc-HYNIC-ASON solution was collected. Chromatographic assay was performed in both solution systems before and after purification to detect the labeling efficacy and radiochemical purity of ^{99m}Tc-HYNIC-ASON, where Xihua I filter paper as a sustentaculum was developed with 85% methanol as a developer.

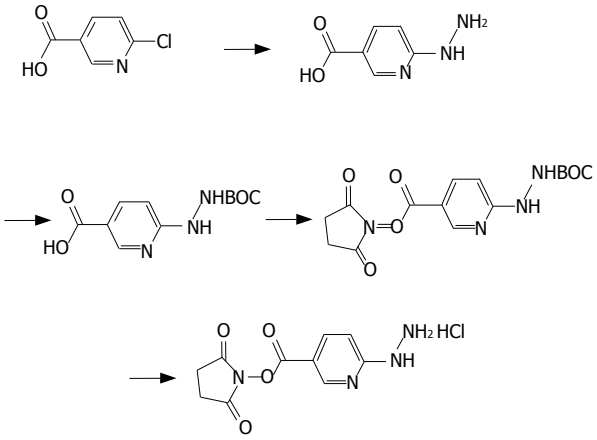

Figure 1 Synthesis of HYNIC.

^{99m}Tc labeling ASON via NHS-MAG_3

Twenty-five microliter ASON (2 mg/mL, dissolved in 0.25 mol/L $NaHCO_3$ and 1 mol/L EDTA, pH = 8.5) was mixed with 42 μL NHS-MAG_3 (10 mg/mL, dissolved in dimethylsulphoxide) at the molar ratio of 1:25. The mixture reacted at room temperature in the dark for 15 min. Any free NHS-MAG_3 was removed through Sep-Pak C18 reversed-phase column (10 mm × 5 mm) in 60% methanol. The bound MAG_3-ASON was collected with a spectrophotometer and frosted to dry powder for storage. The target-bound complex, dry powder on d 15, 30 or 60 at room temperature, was labeled. Fifty μL MAG_3-ASON (1 mg/mL) in re-distilled water was mixed with 10 μL $NaHCO_3$ (0.5 mol/L)-sodium tartrate (50 mg/mL) buffer (pH = 9.2). Ten μL $SnCl_2 \cdot 2H_2O$ fresh solution (1 mg/mL, dissolved in 0.1 mol/L HCl) was dropped into MAG_3-ASON at room temperature and mixed uniformly. At last, 0.2 mL $^{99m}TcO_4^-$ (containing a radioactivity of 370, 740 or 1480 MBq) was added into the above solutions, respectively. After 15 min, ^{99m}Tc-ASON-MAG_3 was purified on Sephadex G25 column (250 mm × 5 mm) in an ammonium acetate solution (0.25 mol/L, pH = 5.2) and collected. Chromatographic assay was performed in both specimens before and after purification to evaluate the labeling efficiency and radiochemical purity. A system was demanded to develop Xihua I filter paper in 85% methanol.

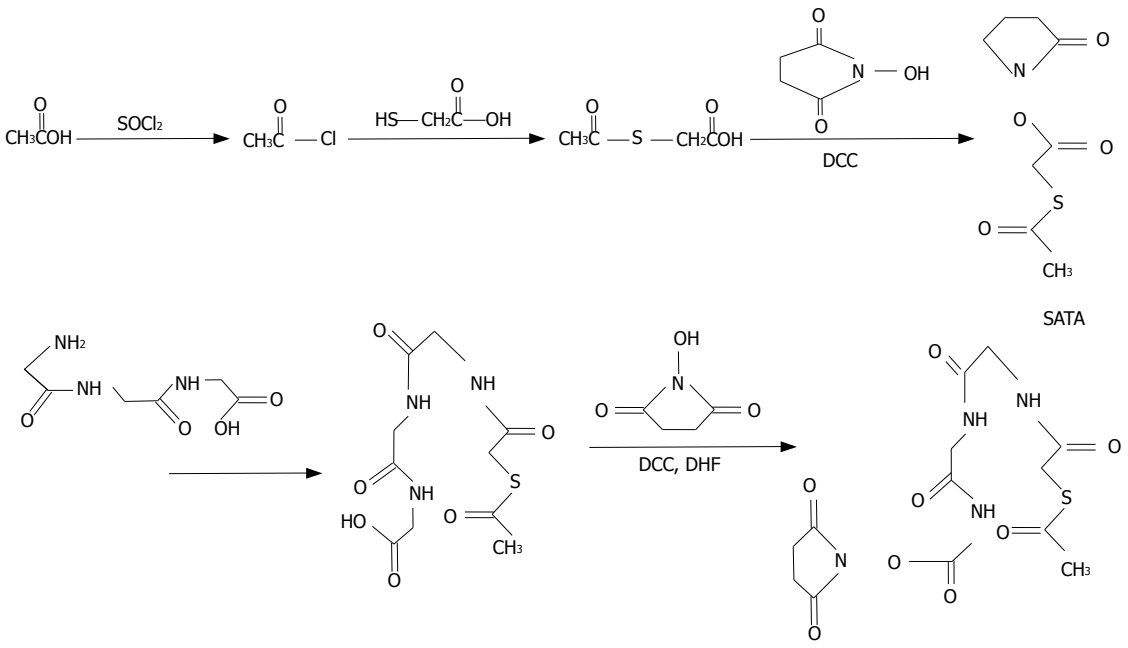

Figure 2 Synthesis of NHS-MAG3. S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) was initially synthesized from acetic acid. S-acetyl MAG3 was obtained by the reaction of SATA and triglycine. NHS-MAG3 was produced with the coupling of S-acetyl MAG3 and N-hydroxybudityrylimine.

Stability of labeled compounds

The stability of labeled compounds was assessed for 1, 2 and 4 h, respectively, at room temperature, by measuring the radiochemical purity on Xihua I filter paper that was developed in 85% methanol.

Test for plasma protein binding in rabbits

Three hundred and seventy mL MBq ^{99m}Tc-HYNIC-ASON or ^{99m}Tc-MAG$_3$-ASON was mixed with 2 mL anti-coagulated rabbit fresh plasma for 6 cuvettes. After incubated at 37 ℃ for 2 h, the mixture was mixed with 5 mL trichloroacetic acid (250 g/L) and centrifuged for 5 min at 1200 × *g*. The precipitate was washed twice with 2 mL trichloroacetic acid (250 g/L) and the supernatant was collected. The radioactivity of precipitate and supernatant was measured, respectively. The binding rate of ^{99m}Tc-HYNIC-ASON or ^{99m}Tc-MAG$_3$-ASON to rabbit plasma protein was calculated by the following formula: Binding rate (%) = radioactivity of precipitate/radioactivity of precipitate and supernatant.

Tissue distribution of labeled compounds in BALB/c mice

^{99m}Tc-HYNIC-ASON or ^{99m}Tc-MAG$_3$-ASON (0.2 mL, 148 KBq) was separately injected into the tail veins of 20 BALB/c nude mice (age: 6-8 wk, body weight: 17-22 g) which were randomly divided into four groups (5 in each group). Mice in each group were sacrificed at 0.5, 1, 2 and 4 h, respectively, after injection of ^{99m}Tc-HYNIC-ASON or ^{99m}Tc-MAG$_3$-ASON. Blood, heart, lungs, liver, kidneys, spleen, stomach, intestine and muscles were removed and weighed. The tissue uptake rate of labeled compounds was calculated according to the following equation: tissue uptake rate (%ID/g) = radioactivity of per gram of wet tissue weight/radioactivity of wet tissue injected into the body. The results were expressed as percentage of radioactivity within per gram of wet tissue.

Cellular uptake of labeled compounds

Human colon carcinoma HT29 cells were incubated with RPMI 1640 medium containing 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Tumor cells were cultured in 80 wells of 96-well plates (1.5×10^6 cells/well). It took about 24 h for cells to adhere to wells. The culture medium was pipetted and 2 mL serum-free RPMI 1640 medium containing 74 KBq ^{99m}Tc-HYNIC-ASON or ^{99m}Tc-MAG$_3$-ASON was added to each of the 80 wells. The cells were incubated in a humidified incubator containing 50 mL/L CO_2 at 37℃ for 10, 20, 40, 60 and 120 min, respectively. Each well was rinsed 3 times with RPMI 1640 medium. At last, all the human colon carcinoma HT29 cells and supernatant in each well were collected and the radioactivity was calculated. The following formula was used to calculate the percentage of radioactivity within the cells of each well: cellular uptake rate (%) = radioactivity absorbed in each well/radioactivity added to each well.

Statistical analysis

The data were expressed as mean ± SD and input into a computer for statistical analysis with SPSS 11 software. Differences among the groups were compared with paired *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Labeling efficiency and radiochemical purity of labeled compounds

Analysis of labeling efficiency and radiochemical purity of the labeled compounds showed that the flow rate of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-MAG$_3$-ASON, $^{99m}TcO_4^-$, and deoxidized technetium, was 0.9-1.0, 0.6-0.7, and 0-0.1, respectively. The labeling efficiency and radiochemical purity of labeled compounds are

Table 1 Labeling efficacy and radiochemical purity of labeled compounds (mean ± SD)

	Interval between binding and labeling (d)			Radioactivity of $^{99m}TcO_4^-$ (MBq)		
	15	30	60	370	740	1480
Labeling efficiency (%)						
Via HYNIC	57.36 ± 3.69	62.13 ± 4.25	62.87 ± 3.04	58.74 ± 5.32	62.86 ± 4.27	63.28 ± 3.38
Via NHS-MAG_3	67.35 ± 4.03	68.35 ± 3.56	69.85 ± 4.63	68.67 ± 4.82	70.31 ± 5.09	71.56 ± 5.37
Radiochemical purity (%)						
^{99m}Tc-HYNIC-ASON	95.75 ± 5.21	96.32 ± 4.92	95.86 ± 5.28	96.56 ± 4.45	96.87 ± 3.65	97.16 ± 4.34
^{99m}Tc-MAG_3-ASON	96.43 ± 4.69	95.67 ± 5.17	96.39 ± 4.78	96.35 ± 6.12	95.86 ± 4.67	96.54 ± 5.65

ASON: Anti-sense oligonucleotide; HYNIC: Hydrazino nicotinamide derivative; NHS-MAG_3: N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycline.

Table 2 Radiochemical stability of labeled compounds (%, mean ± SD)

	Incubation time at room temperature (h)			Incubation time at 37℃ (h)		
	1	2	4	1	2	4
^{99m}Tc-HYNIC-ASON	93.43 ± 5.32	89.17 ± 4.62	87.16 ± 5.36	92.75 ± 4.46	89.52 ± 3.67	86.86 ± 5.49
^{99m}Tc-MAG_3-ASON	97.26 ± 6.02	96.68 ± 5.54	96.39 ± 4.68	95.86 ± 5.69	95.47 ± 4.07	94.79 ± 5.34

Table 3 Biodistribution of labeled compounds in BALB/c mice (% ID/g) (mean ± SD)

Tissue	0.5 h		1 h		2 h		4 h		Paired	*t*-test
	M	H	M	H	M	H	M	H		
Blood	1.12 ± 0.76	6.21 ± 1.03	2.38 ± 0.63	6.56 ± 1.11	1.14 ± 0.42	3.58 ± 1.21	1.10 ± 0.09	2.83 ± 0.54	t = 4.347	P = 0.022
Heart	0.62 ± 0.31	2.13 ± 0.45	0.58 ± 0.07	1.64 ± 0.34	0.32 ± 0.05	1.16 ± 0.12	0.18 ± 0.08	0.81 ± 0.13	t = 5.362	P = 0.013
Lungs	3.11 ± 0.82	2.68 ± 0.65	3.87 ± 1.36	3.23 ± 1.04	3.04 ± 0.79	2.78 ± 1.03	2.08 ± 0.62	1.76 ± 0.36	t = -4.934	P = 0.016
Liver	7.52 ± 2.45	11.46 ± 2.31	13.19 ± 1.47	15.24 ± 2.53	9.21 ± 1.03	12.89 ± 1.68	9.48 ± 2.56	10.46 ± 1.97	t = 3.806	P = 0.032
Kidneys	11.42 ± 3.34	4.17 ± 1.05	17.13 ± 2.86	5.03 ± 0.94	24.58 ± 3.57	2.78 ± 0.68	21.95 ± 4.02	2.28 ± 0.95	t = -4.511	P = 0.020
Spleen	2.71 ± 1.62	4.87 ± 2.36	5.65 ± 0.93	6.08 ± 1.93	5.35 ± 0.26	4.08 ± 1.54	4.84 ± 1.33	5.21 ± 2.04	t = 0.603	P = 0.589
Stomach	1.08 ± 0.86	7.46 ± 2.13	2.58 ± 0.95	11.48 ± 3.01	1.73 ± 0.21	7.49 ± 1.86	1.41 ± 0.34	7.85 ± 3.02	t = 9.901	P = 0.002
Intestines	0.53 ± 0.31	1.26 ± 0.31	0.86 ± 0.14	2.68 ± 0.95	1.23 ± 0.19	6.44 ± 2.13	1.96 ± 0.53	7.84 ± 2.34	t = 2.706	P = 0.073
Muscle	1.35 ± 0.16	0.54 ± 0.21	0.87 ± 0.63	0.94 ± 0.81	0.75 ± 0.08	0.42 ± 0.06	0.64 ± 0.15	0.19 ± 0.05	t = -2.095	P = 0.127

M: ^{99m}Tc-MAG_3-ASON; H: ^{99m}Tc-HYNIC-ASON. Statistical analysis of the same tissue distribution of radioactivity was made after the injection of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-MAG_3-ASON.

listed in Table 1. The labeling efficiency of ^{99m}Tc *via* NHS-MAG_3 was higher than that of ^{99m}Tc *via* HYNIC (for interval between binding and labeling: t = 6.715, P = 0.021; for radioactivity of $^{99m}TcO_4^-$: t = 11.736, P = 0.007). The radioactivity of $^{99m}TcO_4^-$ hardly influenced the labeling efficiency. The radiochemical purity of labeled compounds was higher than 95% and there was no statistical difference between the two methods (for interval between binding and labeling: t = -0.444, P = 0.701; for radioactivity of $^{99m}TcO_4^-$: t = 2.656, P = 0.117). Either interval between binding and labeling of HYNIC-ASON or that of MAG_3-ASON had almost no effect on the labeling efficiency and radiochemical purity of labeled compounds.

Radiochemical purity of labeled compounds

To assess the radiochemical purity, the labeled compounds were incubated at room temperature or at 37℃ after diluted with an equal volume of fresh human serum (Table 2). The radiochemical purity of ^{99m}Tc-MAG_3-ASON was much higher than that of ^{99m}Tc-HYNIC-ASON (at room temperature: t = 5.616, P = 0.030; at 37℃: t = 5.616, P = 0.032), while the radiochemical purity of ^{99m}Tc-MAG_3-ASON was less affected by incubation time than that of ^{99m}Tc-HYNIC-ASON.

Binding rate of rabbit plasma protein

The binding rate of rabbit serum protein for ^{99m}Tc-MAG_3-ASON or ^{99m}Tc-HYNIC-ASON was 11.17% ± 1.31% and 71.06% ± 3.56%, respectively. The differences between them were statistically significant, and the former was lower than the latter (t = 27.346, P < 0.0001).

Biodistribution of ^{99m}Tc-MAG_3-ASON and ^{99m}Tc-HYNIC-ASON in BALB/c mice

The biodistributions of ^{99m}Tc-MAG_3-ASON and ^{99m}Tc-HYNIC-ASON in BALB/c mice are listed in Table 3. The distributions of ^{99m}Tc-MAG_3-ASON were significantly lower in blood, heart, liver and stomach than those of ^{99m}Tc-HYNIC-ASON (P < 0.05). The distributions of ^{99m}Tc-MAG_3-ASON were significantly higher in lungs and kidneys than those of ^{99m}Tc-HYNIC-ASON (P < 0.05).

Table 4 Human colon carcinoma HT29 cellular uptake of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-MAG$_3$-ASON (mean ± SD)

	10 min	20 min	40 min	60 min	120 min
^{99m}Tc-HYNIC-ASON (%)	0.43 ± 0.08	0.56 ± 0.21	0.93 ± 0.54	1.42 ± 0.64	1.67 ± 0.86
^{99m}Tc-MAG$_3$-ASON (%)	2.78 ± 0.81	5.64 ± 0.51	7.82 ± 2.53	13.63 ± 2.71	15.25 ± 3.13

There was no statistical difference in the distributions of ^{99m}Tc-MAG$_3$-ASON and ^{99m}Tc-HYNIC-ASON in spleen, intestines and muscle.

Cellular uptake of labeled compounds

Cellular uptake of labeled compounds in human colon carcinoma HT29 cells is listed in Table 4. The cellular uptake of ^{99m}Tc-MAG$_3$-ASON was significantly higher than that of ^{99m}Tc-HYNIC-ASON (t = 3.770, P = 0.020), which was 6.5, 10.1, 8.4, 9.5 and 9.1-folds higher than those of ^{99m}Tc-HYNIC-ASON at 10, 20, 40, 60 and 120 min after incubation.

DISCUSSION

In our studies, because expensive acetylsulfoacetic acid was not available, S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) was synthesized as previously described[5]. The synergenic coligand of tricine (N-tris-hydroxy-methyl-methylglycine) was applied to the synthesis of HYNIC, to achieve the high radioactivity of labeled compounds. During the synthesis of labeled compounds, isopropylol was used to crystallize the compounds instead of chromatographic column purification. Their synthesis was simple, efficient, economical, with a high yield (75%-80%) and little environmental pollution. Nuclear magnetic resonance of labeled compounds was performed as previously described[5-7]. Both Sep-Pak C18 reversed-phase column and Sephadex G25 column could be used to purify the radiolabeled ASON. Both labeling methods can achieve a high radiochemical purity of over 95%.

Stability can be obtained by methylation, amination or sulfonation of the phosphorus atoms in ASON, making it not recognized and degradated by nucleic acid enzyme[8]. In the present study, we modified the ASON by replacing the hydroxyl group in the phosphoric acid branch of ASON with a sulphur atom and attaching an amid to the 5 ' terminal of ASON. Labeled compounds were observed for four hours to detect the stability of ASON labeled with ^{99m}Tc *via* HYNIC or NHS-MAG$_3$. Only 1-2 covalent bonds were formed between a molecule of HYNIC-ASON and a technetium atom. However, it was reported that 4-5 covalent bonds can form between MAG$_3$-ASON and technetium[9,10], which may be the reason for a greater stability of ^{99m}Tc-MAG$_3$-ASON than that of ^{99m}Tc-HYNIC-ASON. During labeling, since the mercapto group of ASON-MAG$_3$ is protected by acetyl group, excessive $SnCl_2$ is needed to hydrolyze the protection group of ASON-MAG$_3$[11], which may be the reason for a greater labeling efficiency of ^{99m}Tc *via* NHS-MAG$_3$ than *via* HYNIC.

The binding rate of ^{99m}Tc-MAG$_3$-ASON to rabbit serum protein was significantly lower than that of ^{99m}Tc-HYNIC-ASON in our study, suggesting that the distributions of ^{99m}Tc-MAG$_3$-ASON are significantly lower in blood, heart and liver of BALB/c mice. The distributions of ^{99m}Tc-MAG$_3$-ASON were much lower in stomach than those of ^{99m}Tc-HYNIC-ASON, suggesting that ^{99m}Tc-MAG$_3$-ASON has a greater *in vivo* ability than ^{99m}Tc-HYNIC-ASON. The distributions of ^{99m}Tc-MAG$_3$-ASON were much higher in kidneys than those of ^{99m}Tc-HYNIC-ASON, which may be related to the metabolism of MAG$_3$-ASON in kidneys.

Cellular targeting uptake of ASON can be improved by receptor-mediated mechanisms[12-14]. The conjugation of vasoactive intestinal peptide (VIP)-ASON is very helpful for ^{125}I-ASON to selectively bind to HT29 tumor cells by VIP receptors. For such tumor cells that highly express VIP receptors, tumor cellular uptake of VIP-^{125}I-ASON is significantly higher than that of ^{125}I-ASON un-conjugated to VIP[12]. The c-myc ASON complex entered human melanoma cells (M14) by folacin receptors on tumor cell surface, brings about a greater cellular uptake than that of free-ASON, and inhibits tumor growth by lowing c-myc cancer protein expression[13]. As we know, the c-myc oncogene and transferrin receptors are highly expressed in HL-60 and LoVo Dx cells, the addition of transferrin-polylysine-c-myb ASON complex would cause more tumor cell deaths than free c-myb ASON[14]. However, receptor mediation was not used in our study. Why HT29 cellular uptake of ^{99m}Tc-MAG$_3$-ASON is higher than that of ^{99m}Tc-HYNIC-ASON is unclear, which is possibly related to the greater stability of ^{99m}Tc-MAG$_3$-ASON, and needs further study.

COMMENTS

Background

Anti-sense oligonucleotide (ASON) is used to bind to deoxyribonucleic acid (DNA) translation or transcription and interfere with the expression of oncogene. However, ASON is not sufficient to inhibit tumor growth. In order to enhance anti-tumor effect of ASON, we labeled ASON with technetium-99m *via* N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycline (NHS-MAG$_3$) and hydrazinonictinamide derivative (HYNIC).

Research frontiers

Many proteins such as monoclonal antibody, polypeptide, ligand, can be labeled with radionuclides, such as ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{99m}Tc, ^{188}Re, ^{186}Re, ^{90}Y. We are trying to label oncolytic virus with radionuclide, in order to achieve a synergistic anticancer effect.

Innovations and breakthroughs

In this study, we compared the radiochemical behaviors and biological properties of anti-sense oligonucleotide (ASON) labeled with technetium-99m *via* N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycline (NHS-MAG$_3$) and

hydrazinonictinamide derivative (HYNIC) and found that ^{99m}Tc-MAG$_3$-ASON showed superior radiochemical behaviors and biological properties than ^{99m}Tc-HYNIC-ASON.

Applications

^{99m}Tc-MAG$_3$-ASON showed superior radiochemical behaviors and biological properties than ^{99m}Tc-HYNIC-ASON, suggesting that it can be used as a potential radiopharmaceutic agent for *in vivo* application.

Peer review

In this study, the authors analyzed and compared the radiochemical behaviors and biological properties of anti-sense oligonucleotide (ASON) labeled with technetium-99m *via* NHS-MAG$_3$ and HYNIC. The rationale of the study is clearly expressed and the experiments appear to be carefully conducted.

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RAPID COMMUNICATION

Effects of simulated carbon dioxide and helium peumoperitoneum on proliferation and apoptosis of gastric cancer cells

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Abstract

AIM: To investigate the effects of carbon dioxide (CO_2) and helium insufflation administered at different pressures on the growth and apoptosis of cultured human gastric cancer cells.

METHODS: The gastric cancer cells MKN-45 were exposed to a CO_2 and helium environment maintained at different pressures (0, 5, 10 and 15 mmHg). The cells were exposed to simulated pneumoperitoneum environment for 4 h, and pH of the culture media was measured after it was moved to normal conditions for 0, 2, 4, 6 and 8 h. Proliferation viability of MKN-45 was examined by 3-[4,5Dimethylthiazol-2-yl],5-diphenyltetrazolium bromide or triazolyl blue (MTT) assay after it was moved to normal conditions. Apoptotic ratio was measured by Annexin V-FITC/PI double labelled staining.

RESULTS: The pH of media was acid and recovered to normal after 4 h in the CO_2 group while it was basic in the helium group. There was no difference between CO_2 groups (under 10 mmHg) and control group ($P > 0.05$) in the proliferative viability of the cells. The cultured cells exposed to 15 mmHg CO_2 environment grew more slowly than control group from 4 to 7 d ($P < 0.01$) while there was no difference from 1 to 3 d ($P > 0.05$). The proliferative viability in helium group was not obviously different from the control group ($P > 0.05$). The apoptotic ratio of the cultured cells was markedly higher than that of the control group ($P < 0.01$) at 10 and 15 mmHg CO_2 insufflation pressure. In helium group, the apoptotic ratio was not obviously different from the control group ($P > 0.05$).

CONCLUSION: There is no obvious effect in the proliferation and apoptosis of MKN-45 cells under 10 mmHg CO_2 insufflation pressure and helium in any pressure. Fifteen mmHg CO_2 insufflation pressure can inhibit the proliferation of the cells and improve apoptosis.

Key words: Pneumoperitoneum; Gastric cancer cells; Proliferation; Apoptosis

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INTRODUCTION

Minimally invasive techniques are increasingly applied in abdominal surgeries[1,2]. In recent years, numerous authors reported an acceptable feasibility of minimally invasive techniques for biopsy and resection of various malignant tumors[3,4]. However, laparoscopic resection for intraperitoneal malignancies remains controversial. One of the reasons is the concern whether carbon dioxide (CO_2) peumoperitoneum can improve cancer cells' growth[5,6]. There is an ongoing debate about the deleterious effects of CO_2 on tumor cell behavior. Some authors showed an increase in cell proliferation and tumor growth[7] and others found beneficial effects of CO_2 exposition *in vitro* and in animal studies[8,9].

It is well known that intracellular and extracellular pH in the peritoneum is affected by CO_2 insufflation. And some authors reported that pH in peritoneal cavity may be an important regulator of cell functions, such as adenosine

triphosphate (ATP) production, cell proliferation, and apoptosis[10]. Apart from the acid of peritoneal cavity, whether the direct insult of insufflation pressure could affect the growth of tumor cells is unclear.

Therefore, we focused on the different gases and pressures in simulated pneumoperitoneum, and investigated the proliferative viability of gastric cancer cells and apoptotic ratio *in vitro*.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cells (MKN-45; personal gift of Professor F Daiming, Fourth Military Medical University) were cultured in RPMI-1640 (HyClone, USA) culture medium supplemented with 100 g/L fetal bovine serum, penicillin G 100 IU/mL and streptomycin sulfate 100 μg/mL.

Pneumoperitoneum model in vitro

To simulate the environment produced during laparoscopic surgery, we designed an *in vitro* pneumoperitoneum according to Ridgway's method[11]. We used 100% CO_2 or 100% helium as the insufflation gas-displacement model. Sub-confluent MKN-45 cells which had been plated on 6 cm Petri dishes were placed into modified desiccating chambers. CO_2 or helium insufflation was affected by the connection of a standard surgical insufflator (Stryker, USA) to the chamber. Cells were exposed to a continual pneumoperitoneum for 4 h at 0, 5, 10 and 15 mmHg at 37℃. The pH of the media was examined using an arterial blood gas analyzer (Radiometer ABL 505, Denmark). After the cells were exposed to CO_2 or helium for 4 h, the media was changed and the cells were allowed to grow for 24 h before 3-[4,5Dimethylthiazol-2-yl],5-diphenyltetrazolium bromide or triazolyl blue (MTT) assay or flow cytometry.

Cell viability

Cell growth was determined with a spectrophotometric assay[12]. This water-soluble tetrazolium salt was cleaved by the mitochondrion of living cells to an insoluble purple formazan. Optical density readings were measured at 490 nm.

Percentage of apoptotic cells

The percentage of apoptotic cells was determined by FITC-labeled Annexin V and PI double staining flow cytometry.

The cell growth and apoptosis for each group were compared with those of the control group using one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered significant.

RESULTS

Influence of pneumoperitoneum on pH of media

The pH of media in CO_2 and helium group is shown in Tables 1 and 2. When the pressure of CO_2 pneumoperitoneum was 15 mmHg, the pH of media was 6.18. It became normal after 4 h when moved to normal cultured environment. In the helium group, the pH of the media was 8.12 when the pressure was 15 mmHg. Six hours later, it dropped to 7.18 when it was moved to normal cultured environment (Tables 1 and 2).

Table 1 Changes of culture media pH in CO_2 groups (n = 4) (mean ± SD)

Groups	Time (h)				
	0	2	4	6	8
Control	7.20 ± 0.02	7.18 ± 0.02	7.15 ± 0.01	7.16 ± 0.03	7.20 ± 0.02
0 mmHg	7.13 ± 0.04[b]	7.15 ± 0.03	7.15 ± 0.03	7.19 ± 0.01	7.23 ± 0.04
5 mmHg	7.00 ± 0.05[b]	7.13 ± 0.03	7.22 ± 0.02	7.22 ± 0.02	7.24 ± 0.02
10 mmHg	6.77 ± 0.03[b]	6.95 ± 0.05[b]	7.16 ± 0.03	7.22 ± 0.01	7.21 ± 0.01
15 mmHg	6.18 ± 0.02[b]	6.91 ± 0.02[b]	7.08 ± 0.04	7.20 ± 0.02	7.22 ± 0.01

[b]$P < 0.01$ *vs* control group.

Table 2 Changes of culture media pH in helium groups (n = 4) (mean ± SD)

Groups	Time (h)				
	0	2	4	6	8
Control	7.20 ± 0.01	7.18 ± 0.02	7.15 ± 0.01	7.17 ± 0.03	7.20 ± 0.02
0 mmHg	7.42 ± 0.02[b]	7.23 ± 0.03	7.18 ± 0.01	7.15 ± 0.01	7.16 ± 0.03
5 mmHg	7.53 ± 0.03[b]	7.28 ± 0.02[b]	7.21 ± 0.02[a]	7.19 ± 0.03	7.15 ± 0.01
10 mmHg	7.82 ± 0.02[b]	7.31 ± 0.01[b]	7.23 ± 0.02[b]	7.20 ± 0.05	7.15 ± 0.02
15 mmHg	8.19 ± 0.04[b]	7.96 ± 0.03[b]	7.33 ± 0.05[b]	7.22 ± 0.01	7.19 ± 0.02

[a]$P < 0.05$ *vs* control group, [b]$P < 0.01$ *vs* control group.

MTT assay

According to MTT chromometry, the proliferative viability of MKN-45 cells was significantly decreased from d 4 to d 7 after it was exposed to simulated CO_2 pneumoperitoneum at 15 mmHg. When the pressure was under 10 mmHg, the cells' proliferative viability was not obviously different from the control group ($P > 0.05$). In the helium group, there was no difference between various pressures and control group ($P > 0.05$), even at 15 mmHg (Tables 3 and 4).

Percentage of apoptotic cells

The percentage of apoptotic cells in 10 and 15 mmHg CO_2 groups was significantly higher than control group ($P < 0.01$). In the helium group, there was no significant difference in the percentage of apoptotic cells under different pressures ($P > 0.05$). Even the pressure was 15 mmHg, there was no significant difference from the control group ($P > 0.05$) (Table 5).

DISCUSSION

Several prospective, randomized studies on laparoscopically assisted surgeries for early gastric cancer have demonstrated that the 5-year survival of patients with laparoscopically assisted radical resection of gastric carcinomas was similar to or even higher than that of open surgery[13]. Since March 2004, we have performed 304 cases of laparoscopically

Table 3 Changes of MKN-45 proliferative viability in CO_2 groups (OD, mean ± SD)

Groups	Time (d)						
	1	2	3	4	5	6	7
Control	0.31 ± 0.04	0.41 ± 0.02	0.53 ± 0.09	1.38 ± 0.04	1.81 ± 0.09	2.33 ± 0.04	2.33 ± 0.06
0 mmHg	0.28 ± 0.06	0.41 ± 0.04	0.56 ± 0.06	1.37 ± 0.07	1.58 ± 0.02	2.38 ± 0.06	2.39 ± 0.08
5 mmHg	0.31 ± 0.05	0.35 ± 0.05	0.56 ± 0.04	1.34 ± 0.04	1.59 ± 0.07	2.50 ± 0.07	2.32 ± 0.04
10 mmHg	0.29 ± 0.02	0.36 ± 0.04	0.53 ± 0.05	1.27 ± 0.05	1.57 ± 0.14	2.54 ± 0.10	2.40 ± 0.03
15 mmHg	0.32 ± 0.03	0.39 ± 0.05	0.47 ± 0.05	0.68 ± 0.04[b]	0.80 ± 0.04[b]	1.16 ± 0.08[b]	1.42 ± 0.02[b]

[b]$P < 0.01$ *vs* control group; OD: Optical density.

Table 4 Changes of MKN-45 proliferative viability in helium groups (OD, mean ± SD)

Groups	Time (d)						
	1	2	3	4	5	6	7
Control	0.29 ± 0.04	0.36 ± 0.04	0.58 ± 0.03	1.22 ± 0.05	1.83 ± 0.03	2.21 ± 0.04	2.62 ± 0.04
0 mmHg	0.30 ± 0.02	0.39 ± 0.02	0.60 ± 0.04	1.23 ± 0.06	1.86 ± 0.06	2.37 ± 0.05	2.64 ± 0.05
5 mmHg	0.30 ± 0.02	0.38 ± 0.03	0.58 ± 0.03	1.27 ± 0.05	1.80 ± 0.08	2.40 ± 0.33	2.75 ± 0.12
10 mmHg	0.31 ± 0.03	0.41 ± 0.03	0.57 ± 0.05	1.25 ± 0.06	1.78 ± 0.04	2.49 ± 0.26	2.71 ± 0.18
15 mmHg	0.33 ± 0.02	0.39 ± 0.04	0.54 ± 0.09	1.24 ± 0.23	1.81 ± 0.09	2.31 ± 0.20	2.73 ± 0.11

OD: Optical density.

Table 5 Changes of MKN-45 apoptosis ratio in CO_2 and helium groups (%, mean ± SD)

Groups	Control	0 mmHg	5 mmHg	10 mmHg	15 mmHg	F
CO_2	0.21 ± 0.02	0.19 ± 0.04	0.29 ± 0.05	9.20 ± 0.44[a]	11.60 ± 0.95[a]	430.09
He	0.28 ± 0.04	0.27 ± 0.04	0.31 ± 0.08	0.35 ± 0.11	0.37 ± 0.05	0.99

[a]$P < 0.05$ *vs* control group; CO_2: Carbon dioxide; He: Helium.

assisted gastrectomy, 236 of the cases were advanced gastric cancer. We found no obvious difference between excising tumor with tumor-free margin and dissecting lymph nodes radically[14,15]. However, laparoscopic resection for abdominal malignancy remains controversial, especially for advanced gastric cancer. Among the reasons for this is the concern whether CO_2 pneumoperitoneum can improve port-site metastasis, peritoneal dissemination and recurrence[5,16,17].

The results of experimental studies on the behavior of tumor cells exposed to CO_2 are not conclusive. Numerous authors confirmed a CO_2 associated increase of tumor growth and invasiveness of various cell lines derived from colon carcinoma, adenocarcinoma, and other tumors using animal models[18-20]. However, other studies showed that CO_2 pneumoperitoneum could increase cell necrosis and decrease proliferation[8,21]. Our data indicated that the exposure to CO_2 decreased the mitochondrial activity of MKN-45 cells, especially in a higher pressure environment (15 mmHg). We noticed this change when it was moved to normal culture environment for 4 h. The percentage of apoptotic cells increased in CO_2 pneumoperitoneum (10 and 15 mmHg group). This phenomenon was also investigated in human ovarian cancer cell lines HO8901, $SKVO_3$[22] and other tumor cells[23,24].

Helium has been suggested for alternative use for pneumoperitoneum to prevent CO_2 effects such as local acidosis and systemic hypercapnia[25]. In addition, a beneficial effect of helium versus CO_2 on the growth of rat mammary adenocarcinoma cells was shown *in vitro*[26]. In our experiments, we observed no obvious difference between helium group and control group, even at 15 mmHg pressure. This proved that the increase of cell apoptotic ratio in CO_2 pneumoperitoneum might not only depend on insufflated pressure.

Kos *et al* showed that intracellular and extracellular acidification associated with CO_2 resulted in an attenuation of cytokine release and cell activity in macrophages[27]. Takiguchi *et al* believed CO_2 pneumoperitoneum had no effect on cancer cells' proliferative ratio but had a toxic effect on cancer cells[18].

Our current experiments confirmed that the extracellular pH differed significantly between CO_2 and helium exposure and it decreased very sharply at the insufflated pressure. Wildbrett *et al* reported that intracellular and extracellular pH and calcium level were altered with CO_2 pneumoperitoneum[10]. pH and calcium are important regulators of cell functions such as ATP production, cell cycle, intracellular signaling and apoptosis[28,29]. It is likely that all these changes influence the favorability of tumor-cell implantation at the time of laparoscopic surgery.

West *et al* excluded hypoxia as a cause of alteration of cell functions by exposing cells to 20% and 80% CO_2[30]. In our experiments, exposition to both 100% CO_2 and 100% helium may cause hypoxia, but the impact on MKN-45 gastric cancer cells was significantly different. Only CO_2 reduced cell activity, which made no hypoxic effects.The direct effects of CO_2 demonstrated by Takiguchi *et al* on human colon cancer cells *in vitro*[18] remain to be confirmed for gastric cancer cells.

CO_2 pneumoperitoneum resulted in severe peritoneal

acidosis, and peritoneal acidosis may play a role in changing tumor cells' implantation during laparoscopic oncologic surgery. The role of peritoneal microenvironment in tumor-cell growth awaits further studies. More studies in the area could enable us to find the safest approach to laparoscopic oncologic surgery.

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COMMENTS

Background

Laparoscopic surgery in oncologic patients is increasingly adopted as an alternative to conventional surgical procedures, both for diagnosis and resection. However, some experimental and clinical studies have suggested that the CO_2 pneumoperitoneum influences the development of intra-abdominal tumor dissemination and port site metastases. Numerous authors confirmed a CO_2 associated increase of tumor growth and invasiveness of various cell lines derived from colon carcinoma, adenocarcinoma, and other tumors. However, other studies showed beneficial effects of CO_2, such as increased cell necrosis and decreased proliferation.

Research frontiers

The effects of laparoscopic environment on tumor cell biology, including the kind of gas and the pressure of pneumoperitoneum.

Innovations and breakthroughs

The results of experimental studies on the behavior of tumor cells exposed to CO_2 are not conclusive. In this study, the authors elaborately and clearly demonstrate that it is the CO_2 gas and not the pressure or the hypoxia that inhibits the growth of the cancer cells and increases apoptosis.

Applications

Laparoscopic resection for intra-abdominal malignancies remains controversial, especially for advanced gastric cancer. One of the reasons is the concern whether CO_2 pneumoperitoneum can improve port-site metastasis, peritoneal dissemination and recurrence. This research on CO_2 pneumoperitoneum could improve the application of CO_2 as the insufflation gas in laparoscopic surgery.

Terminology

Helium insufflation: The act of blowing helium into any body cavity for experimental, diagnostic, or therapeutic purposes. CO_2 pneumoperitoneum: The presence of CO_2 in the peritoneal cavity. It may occur spontaneously or be deliberately introduced as an aid to operate.

Peer review

This is a good study. As far as the *in vitro* effects of gases and pressure on cancer cell growth and apoptosis is concerned, one can find studies reporting exactly contradictory findings. The authors elaborately and clearly demonstrate that it is the CO_2 gas and not the pressure or the hypoxia that inhibits the growth of the cancer cells and increases apoptosis.

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RAPID COMMUNICATION

Dynamic changes of IL-2/IL-10, sFas and expression of Fas in intestinal mucosa in rats with acute necrotizing pancreatitis

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Abstract

AIM: To investigate dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with acute necrotizing pancreatitis. To explore the expression of Fas in intestinal mucosa of rats with acute necrotizing pancreatitis (ANP).

METHODS: A total of 64 Sprague-Dawley (SD) rats were randomly divided into two groups: normal control group (C group), ANP group (P group). An ANP model was induced by injection of 50 g/L sodium taurocholate under the pancreatic membrane. Normal control group received isovolumetric injection of 9 g/L physiological saline solution using the same method. The blood samples of the rats in each group were obtained via superior mesenteric vein to measure levels of IL-2, IL-10, sFas and calculate the value of IL-2/IL-10. The levels of IL-2, IL-10 and sFas were determined by ELISA. The severity of intestinal mucosal injury was evaluated by pathologic score. The expression of Fas in intestinal mucosal tissue was determined by immunohistochemistry staining.

RESULTS: Levels of serum IL-2 were significantly higher in P group than those of C group (2.79 ± 0.51 *vs* 3.53 ± 0.62, 2.93 ± 0.89 *vs* 4.35 ± 1.11, 4.81 ± 1.23 *vs* 6.94 ± 1.55 and 3.41 ± 0.72 *vs* 4.80 ± 1.10, respectively, $P < 0.01$, for all) and its reached peak at 6 h. Levels of serum IL-10 were significantly higher in P group than those of C group at 6 h and 12 h (54.61 ± 15.81 *vs* 47.34 ± 14.62, 141.15 ± 40.21 *vs* 156.12 ± 43.10, 89.18 ± 32.52 *vs* 494.98 ± 11.23 and 77.15 ± 22.60 *vs* 93.28 ± 25.81, respectively, $P < 0.01$, for all). The values of IL-2/IL-10 were higher significantly in P group than those of C group at 0.5 h and 2 h (0.05 ± 0.01 *vs* 0.07 ± 0.02 and 0.02 ± 0.01 *vs* 0.03 ± 0.01, respectively, $P < 0.01$, for all), and it were significantly lower than those of C group at 6 h (0.05 ± 0.02 *vs* 0.01 ± 0.01, $P < 0.01$) and returned to the control level at 12 h (0.04 ± 0.01 *vs* 0.05 ± 0.02, $P > 0.05$). In sFas assay, there was no significant difference between P group and C group (3.16 ± 0.75 *vs* 3.31 ± 0.80, 4.05 ± 1.08 *vs* 4.32 ± 1.11, 5.93 ± 1.52 *vs* 5.41 ± 1.47 and 4.62 ± 1.23 *vs* 4.44 ± 1.16, respectively, $P > 0.05$, for all). Comparison of P group and C group, the pathological changes were aggravated significantly in P group. Immunohistochemistry staining show the expression of Fas was absent in normal intestinal tissues, however, it gradually increased after induction of pancreatitis in intestinal tissue, then reached their peaks at 12 h.

CONCLUSION: Fas were involved in the pathogenesis of pancreatitis associated intestinal injury. The mechanisms of Fas may be associated to Fas mediated T helper cell apoptosis.

Key words: Acute necrotizing pancreatitis; Fas; Intestinal mucosal injury; T helper cell

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INTRODUCTION

Acute pancreatitis (AP) is sudden inflammation of the pancreas that may be mild or life threatening but that usually subsides. Although usually self-limiting, 10% to 20% of afflicted patients will progress to acute necrotizing pancreatitis (ANP)[1,2]. The mortality rate among patients with ANP may approach 30% when they progress to multiple organ failure (MOF)[3]. It is generally accepted that AP is often complicated by intestinal injury. Failure of intestinal barrier function often occurs in this condition, resulting in the increased intestinal permeability. It is clear that increased intestinal permeability and bacteria with

or without endotoxin translocation plays a key role in the development of severe complications such as systemic inflammatory response syndrome (SIRS), sepsis, multiple organ dysfunction syndrome (MODS) and MOF[4-7]. However, its pathogenesis remains unclear.

The Fas system was originally characterized as a key mechanism for inducing apoptosis in immune cells, but later it transpired to be very common in various tissues such as liver, ovary, kidney, and testis, especially under I/R conditions. Apoptosis is a teleologically beneficial form of cell death in AP. However, little is known about how the induction of apoptosis reduces the severity of AP[8]. Recent research has demonstrated that a Th1 to Th2 immune deviation is beneficial to ANP. The antigen-induced deletion of Th is often accompanied by an imbalance in Th1 and Th2. The two most polarized patterns of cytokine production, Th1 (characterized by production of IL-2, IFN-γ, TNF-α, and TNF-β) and Th2 (characterized by production of IL-4, 5, 6, 10, and -13) were reported[9].

In the present study, we performed immunohistochemistry staining of apoptosis-related protein Fas and investigated dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with ANP.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats of both sexes weighing 250-300 g were provided by the Laboratory Animal Center of Jiangsu University. The animals were fed with standard rat chow and water *ad libitum*. The rats were allowed to acclimatize to our laboratory conditions for 1 wk and then subjected to mesh stainless-steel cages at a constant temperature (21 ± 1°) in a 12 h day/night cycle. The animals were fasted for 12 h before the experiments but had free access to water. Animal care and experimental procedure were performed in accordance with the guidelines for Animal Experimentation of Jiangsu University with the approval of the Institutional Animal Care and Use Committee.

Experimental design

The animals were randomly divided into: control group (C group), ANP group (P group) with 32 rats in each group. Each group was further divided into 0.5, 2, 6 and 12 h subgroups, respectively. The mortality in the present series was not calculated. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The rats were infused with sodium taurocholate (4 mL/kg, Na-Tc, Sigma) the pancreatic membrane to induce ANP model as previously described[10]. After 30 min, pancreatic edema and dotted bleeding occurred. Normal control group received isovolumetric injection of 9 g/L physiological saline solution using the same method. Animals in each group were sacrificed at 0.5, 2, 6 and 12 h after infusion for further examination. Part of distal ileum and pancreas were removed immediately and fixed in paraformaldehyde solution for 12-24 h and paraffin-embedded for routine histopathologic analysis. The histopathologists were blinded to routine histopathologic analysis.

Analysis of Th1/Th2 cytokines and sFas

The blood of rats in each group was obtained via superior mesenteric vein for determination of serum IL-2, L-10 and sFas levels at 0.5, 2, 6 and 12 h after infusion. Serum levels of IL-2, L-10 and sFas were measured by double antibody sandwich ELISA according to the manufacturer's protocol (Shanghai Senxiong Technology Enterprise Co., Ltd.). The optical density of each well was determined within 30 min using a microplate reader (492 nm).

Pathological examination

The whole pancreas and parts of distal ileum were obtained and promptly fixed in 40 g/L phosphate-buffered formaldehyde for further studies. Paraffin-embedded tissue sections (5 μm thick) were stained with hematoxylin and eosin. Mucosal damage was assessed according to the standard scale of Chiu *et al*[11]. Grading was performed and classified as 0 = normal mucosa; 1 = development of subepithelial space at the tip of the villus; 2 = extension of the space with epithelial lifting; 3 = massive epithelial lifting; 4 = denuded villi; 5 = disintegration of the lamina propria.

Immunohistochemistry

After embedding in paraffin, sections 5 μm in thickness were immersed twice into xylene for 5 min each, followed by immersion twice for 3 min each in 100% ethanol and then 95% ethanol. Slides were rinsed for 30 sec using deionized water and then immersed twice in deionized water for 5 min. To detect Fas expression, heat-induced Ag retrieval was performed using 0.01 mol/L citrate buffer (pH 6.2) and 10 min slide immersion into 95℃ waterbath. Immunoenzyme double staining of intestinal tissue was performed using DAKO EnVision Doublestain System. The sections were then counterstained using hematoxylin before study.

Statistical analysis

All data were analyzed with the SPSS 11.0 software. The results were expressed in mean ± SD except for date on the grading of intestinal mucosal lesions. Differences of grading of intestinal mucosal lesions were determined using the non-parametric Mann-Whitney test. Statistical analysis was performed with post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Serum IL-2 level

At 0.5 h after injection of 50 g/L sodium taurocholate, serum IL-2 level in the samples from mesentery vein in P group were higher than those in C group. From 0.5 h, there was a significant difference between P group and C group ($P < 0.01$, Table 1).

Serum IL-10 level

Upon stimulation, serum IL-10 level was significantly increased in the P group as compared with that of C group at 6 and 12 h ($P < 0.01$, Table 1). There was no significant difference between P group and C group at 0.5 and 2 h.

Table 1 IL-2 and IL-10 level in each group (mean ± SD, n = 8) (pg/mL)

Group		0.5 h	2 h	6 h	12 h
IL-2	C	2.79 ± 0.51	2.93 ± 0.89	4.81 ± 1.23	3.41 ± 0.72
	P	3.53 ± 0.62[b]	4.35 ± 1.11[b]	6.94 ± 1.55[b]	4.80 ± 1.10[b]
IL-10	C	54.61 ± 15.81	141.15 ± 40.21	89.18 ± 32.52	77.15 ± 22.60
	P	47.34 ± 14.62	156.12 ± 43.10	494.98 ± 11.23[b]	93.28 ± 25.81[b]

[b]$P < 0.01$ *vs* control group.

Table 2 Serum IL-2/IL-10 AND Serum sFas in each group (mean ± SD, n = 8)

Group		0.5 h	2 h	6 h	12 h
IL-2/IL-10	C	0.05 ± 0.01	0.02 ± 0.01	0.05 ± 0.02	0.04 ± 0.01
	P	0.07 ± 0.02[b]	0.03 ± 0.01[b]	0.01 ± 0.01[b]	0.05 ± 0.02
sFas (pg/mL)	C	3.16 ± 0.75	4.05 ± 1.08	5.93 ± 1.52	4.62 ± 1.23
	P	3.31 ± 0.80[a]	4.32 ± 1.11[a]	5.41 ± 1.47[a]	4.44 ± 1.16[a]

[a]$P > 0.05$ *vs* control group, [b]$P < 0.01$ *vs* control group.

Table 3 Intestinal mucosal injury in each group (n = 8)

Group	0.5 h						2 h						6 h						12 h					
	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5
C	7	1	0	0	0	0	6	2	0	0	0	0	7	1	0	0	0	0	6	2	0	0	0	0
P	0	2	5	1	0	0[b]	0	0	2	4	2	0[b]	0	0	0	1	4	3[b]	0	0	0	0	3	5[b]

[b]$P < 0.01$ *vs* C group.

Serum IL-2/IL-10

As shown in Table 2, the values of IL-2/IL-10 were higher significantly in P group than those of C group at 0.5 h and 2 h, and it were significantly lower than those of C group at 6 h ($P < 0.01$) and returned to the control level at 12 h ($P > 0.05$) (Table 2) .

Serum sFas level

In serum sFas assay as illustrated Table 2, there was no significant difference between P group and C group. sFas levels were moderate at 0.5 h, peaked at 6 h and decreased at 12 h.

Pathologic examination of pancreas and intestinal mucosa

After induction of ANP model, pancreas showed mild edema and congestion. 2 h after introduction of the model, typical pathologic changes were found in P group, such as a large number of inflammatory cells, necrosis of adjacent fat tissues, interstitial edema, parenchyma hemorrhage and necrosis, large amount of ascites. The degree of intestinal pathological injury is shown in Table 3. The grades of P group were significantly higher those of control group ($P < 0.01$).

Immunohistochemistry

We detected the expression of Fas on intestinal tissue with immunocytochemical technique. Immunohistochemistry staining showed Fas expression in intestinal tissue was absent in normal intestinal tissue, Fas expression in intestinal tissue gradually increased 0.5 h after induction of pancreatitis, and then reached a peak at 12 h (Figure 1).

DISCUSSION

The Fas system is a widely recognized apoptotic signal transduction pathway in which a ligand-receptor interaction triggers the cell death pathway[12]. The Fas system has been implicated in the control of the immune response and inflammation, the response to infection and death of parenchymal cells in several organs[13-15], which is involved in maintaining homeostasis in various systems, including maintenance of peripheral T cell[16]. Recent research has demonstrated that a Th1 to Th2 immune shift is beneficial to mucosal immunity[17,18]. A key component of the mucosal immune defense against pathogens is mediated by CD4+ T lymphocytes that can differentiate into functionally distinct subsets[19]. Whereas T-helper 1 (Th1) cells secrete the cytokines IL-2, IFN-γ, TNF-α, and TNF-β, Th2 cells secrete IL-4, 5, 6, 10, and 13. In the current study, we used IL-2 levels as a marker of Th1 response and IL-10 as a marker of Th2 response. In this study, the role of the Fas-mediated cell death pathway in intestinal mucosal injury models was assessed and dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with ANP were also investigated.

According to the result of immunohistochemistry, Fas had a lower expression in intestinal tissue in the P group at 0.5 h and higher expression after 2, 6 and 12 h. In the C groups, Fas was not detected in any part of intestinal tissue. Previous study has shown that IL-10 is a kind of important anti-inflammatory cytokine and plays a role of self-defense mechanism, limiting the intensity of inflammatory process[20-23]. However, the effect of IL-10 level in the course of acute pancreatitis is still not clear[24-26]. IL-10 is a powerful Th2 cell cytokine produced by lymphoid cells. A marked activation of immune system may be observed in patients with AP, being balanced between pro- and anti-inflammatory cytokines in patients with mild but not severe AP. A reduced functional reserve for the synthesis of IL-10 may be observed in patients with severe AP, which might lead to a worst prognosis[27-30].

From the results of this study, it was found that the release of IL-10 was significantly increased in the P group as compared with that of C group at 6 and 12 h. Serum IL-2 level in the P group were higher than those in C group. From 0.5 h, there was a significant difference between P group and C group. The IL-2/IL-10 ratio was significantly increased in the P group as compared with that of C group

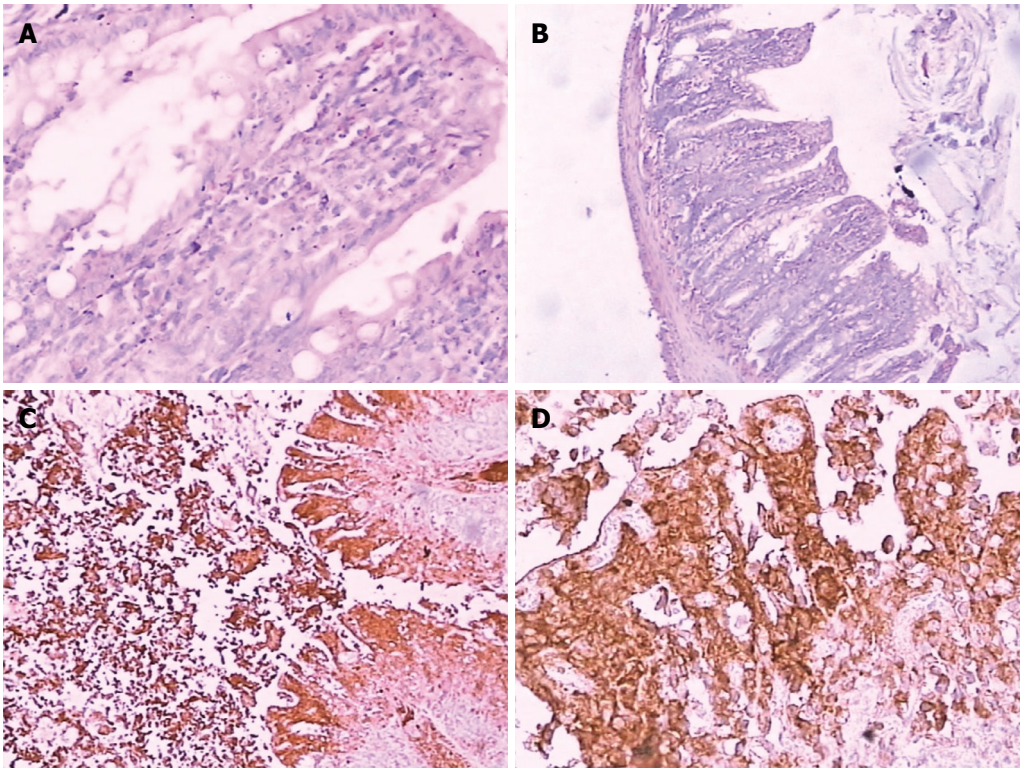

Figure 1 Morphological changes intestinal mucosa after induction of ANP. **A**: Intestinal section with normal mucosa (ANP 0.5 h); **B**: Disintegration of the lamina propria (ANP 6 h); **C**: Fas positive immunohistochemical staining was present in the intestinal mucosa (ANP 0.5 h); **D**: Fas positive immunohistochemical staining was present in the intestinal mucosa (ANP 12 h).

at 0.5 and 2 h, suggesting that a pro-inflammatory response was predominant in these rats and significantly lower in P group at 6 h suggesting that an anti-inflammatory response was predominant. In the sFas assay, there was no significant difference between P group and C group. Interestingly, IL-2, IL-10 and sFas levels were moderate at 0.5 h, peaked at 6 h and decreased at 12 h. In our model, the intestinal tissue injury which was assessed according to the standard scale of pathological examination was closely paralleled by Fas expression and dynamic changes of IL-2/IL-10.

In conclusion, the abnormal apoptosis of Fas can significantly affect the cytokine. Fas were involved in the pathogenesis of intestinal injury in ANP. The mechanisms of Fas may have been related to Fas mediated T helper cell apoptosis.

COMMENTS

Background

Acute pancreatitis (AP) is sudden inflammation of the pancreas that may be mild or life threatening but that usually subsides. It is generally accepted that AP is often complicated by intestinal injury. The Fas system was originally characterized as a key mechanism for inducing apoptosis in immune cells. Apoptosis is a teleologically beneficial form of cell death in AP. However, little is known about how the induction of apoptosis reduces the severity of AP. Recent research has demonstrated that a Th1 to Th2 immune deviation is beneficial to mucosal immunity.

Research frontiers

Recent research has demonstrated that a Th1 to Th2 immune deviation is beneficial to mucosal immunity. The antigen-induced deletion of Th is often accompanied by an imbalance in Th1 and Th2.

Innovations and breakthroughs

In the present study, we performed immunohistochemistry staining of apoptosis-related protein Fas and investigated dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with ANP.

Applications

To provide the experimental basis by expression of Fas in intestinal mucosa in rats with ANP and provide experimental evidence for the immune treatment of patients with ANP.

Terminology

Th1 and Th2 response: Th1-type cytokines tend to produce the proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses. Interferon gamma is the main Th1 cytokine. Excessive proinflammatory responses can lead to uncontrolled tissue damage, so there needs to be a mechanism to counteract this. The Th2-type cytokines include interleukins 4, 5, 13 and interleukin-10, which has more of an anti-inflammatory response.

Peer review

This is a potentially interesting study to understand the dynamic changes of several cytokines and sFas in rats with ANP and provide experimental evidence for the immune treatment of ANP patients.

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RAPID COMMUNICATION

Clinicopathologic characteristics of intrahepatic cholangiocarcinoma in patients with positive serum a-fetoprotein

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Abstract

AIM: To explore clinicopathologic characteristics of intrahepatic cholangiocarcinoma (ICC) in patients with positive serum a-fetoprotein (AFP).

METHODS: One hundred and thirty one patients who underwent surgical dissection for pathologically confirmed ICC were divided into a positive AFP (> 20 ng/mL) group (n = 32) and a negative AFP group (n = 99), whose clinicopathologic features were analyzed and compared.

RESULTS: The positive rate of HBsAg and liver cirrhosis of the positive AFP group was higher than that of the negative AFP group, while the positive rate of CA19-9 (> 37 U/mL) and the lymph node metastasis rate was lower.

CONCLUSION: ICC patients with positive AFP share many clinicopathologic similarities with hepatocellular carcinoma.

Key words: Intrahepatic cholangiocarcinoma; A-fetoprotein; Hepatitis B virus; Liver cirrhosis; Hepatic stem cells

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a tumor originating from peripheral intrahepatic biliary epithelia, ranking as the second most common primary hepatic malignant tumor next to hepatocellular carcinoma (HCC), accounting for 5% of all primary hepatic malignant tumors[1]. The incidence and mortality of ICC is on the rise in recent years[2]. Serum a-fetoprotein (AFP), as a tumor marker of HCC[3-5], and carbohydrate antigen 19-9 (CA19-9), as a tumor marker of ICC, have been widely used in clinical practice[6]. In about 19% ICC patients, serum AFP is also positive (> 20 ng/mL)[1], but there is little knowledge about the clinicopathologic features of such patients. The purpose of this study was to define clinicopathologic features of ICC patients with positive AFP by comparing them with ICC patients with negative AFP.

MATERIALS AND METHODS

Patients

Included in this study were 131 ICC patients who received surgical dissection at the Eastern Hepatobiliary Surgery Hospital of the Second Military Medical University (Shanghai, China) from March 2002 to June 2003, including 90 males and 41 females ranging in age from 23 to 73 years with a mean of 53 years. Of the 131 ICC patients, serum AFP was positive in 32 patients (24.4%), of whom AFP was > 200 ng/mL in 13 patients (9.9%), and > 1000 ng/mL in 6 patients (4.5%). Their clinical manifestations, pathological findings and surgical outcomes were compared with those of ICC patients whose serum AFP was negative. Positive serum hepatitis B surface antigen (HBsAg) and hepatitis C antibody were biomarkers of chronic viral hepatitis.

The diagnosis of ICC was confirmed by pathology. All the excised specimens were fixed in 4% neutral formaldehyde routinely, paraffin embedded, sliced into 4 μm sections, and haematoxylin and eosin (HE) stained.

Table 1 Clinical features of ICC patients with positive AFP

	AFP		
	+ (n = 32)	- (n = 99)	P value
Gender (M/F)	24/8	66/33	NS
Age (yr)	48.7 ± 11.7	54.5 ± 9.9	0.007
HBsAg + (%)	25 (78.1)	38 (38.3)	0.000
Anti-HCV + (%)	0	1 (0.01)	NS
CA19-9 (> 37 U/mL) (%)	10 (31.4)	58 (58.6)	0.007
TBIL (> 17.1 μmol/L) (%)	17 (53.1)	39 (39.4)	NS
ALT (> 40 IU/L) (%)	16 (50.0)	26 (26.3)	0.012
AST (> 40 IU/L) (%)	14 (43.8)	23 (23.2)	0.025

NS: Not significant; M: Male; F: Female; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus antibody; TBIL: Total bilirubin; AST: Aspartate transaminase; ALT: Alanine transaminase.

Pathological study included the size, number and location of the tumors, background of cirrhosis, portal or hepatic vein invasion, lymph node metastasis, formation of tumor capsules, and histological grade. Tumors whose diameter was smaller than 3 cm were classified as small ICC.

Immunohistochemistry for HCC marker hepatocyte paraffin 1 (Hep Par 1)[7] and ICC marker cytokeratin 19 (CK-19)[8] was performed using a polymer-based method with the Envision Kit (Fuzhou Maxim Biotech, China). Formalin-fixed, paraffin-embedded serial tissue sections (4 μm) were deparaffinised and rehydrated in xylene and grade-diluted ethanol. Tissue sections were then incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 min to block endogenous peroxidase. Sections were then incubated overnight at 4℃ with anti-Hep Par 1 antibody (Dako, Denmark) or anti- CK-19 antibody(NeoMarkers, USA), followed by incubation with Envision reagent at room temperature for 30 min, and color developed with 3, 3'-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with haematoxylin, and hyalinized water. For negative controls, the sections were processed the same way, except they were incubated with phosphate-buffered saline instead of the primary antibody.

All patients were followed up after discharge from the hospital, with a median follow-up period of 31 mo (range 5-52 mo).

Statistical analysis

Data were analyzed with SPSS 11.0 statistical software. Quantitative inter-group comparison was tested by t test, and classification inter-group comparison was tested by χ^2 test. Survival analysis was done by Kaplan-Meier method. Inter-group comparison was done by log-rank method. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical features

The mean age of the positive AFP group was lower than that of the negative AFP group ($P = 0.007$). There was no significant difference in sex distribution between the two groups. The positive rate of HBsAg (78.1%) and transaminase of the positive AFP group was higher than that of the negative AFP group ($P = 0.000$ and $P = 0.036$ respectively), while the positive rate of CA19-9 was lower (> 37 U/mL, $P = 0.007$; Table 1).

Table 2 Pathologic features of ICC patients with and without positive AFP

	AFP		
	+ (n = 32)	- (n = 99)	P value
Tumor location (%)			NS
Right lobe	21 (65.6)	57 (57.6)	
Left lobe	8 (25.0)	33 (33.3)	
Both lobes	3 (9.4)	9 (9.1)	
Tumor size (cm)			NS
mean ± SD	7.97 ± 4.12	6.83 ± 2.98	
≤ 3 (%)	2 (0.6)	9 (0.9)	
Tumor number (%)			NS
Single	25 (78.2)	62 (62.7)	
≥ 2	7 (21.8)	37 (37.3)	
Liver cirrhosis (%)	13 (40.6)	22 (22.2)	0.041
Capsule formation (%)	8 (25.0)	15 (15.2)	NS
Histological grades (%)			NS
Well- Moderately	21 (65.6)	68 (68.6)	
Poorly	11 (34.4)	31 (31.4)	
Lymph node metastasis (%)	5 (15.6)	35 (35.4)	0.035
Portal invasion (%)	1 (3.1)	5 (5.1)	NS
Microvascular invasion (%)	18 (56.2)	62 (62.6)	NS
Immunohistochemical examinations			NS
Hepatocyte paraffin	1	0	0
Cytokeratin 19 (%)	32 (100)	99 (100)	

NS: Not significant.

Pathological features

Backgrounds of liver cirrhosis were elicited in 13 ICC patients with positive AFP, which was significantly higher than that of the negative AFP group (40.6% *vs* 22.2%, $P = 0.041$), but the lymph node metastasis rate was significantly lower (15.6% *vs* 35.4%, $P = 0.035$). There were no significant differences in the location, size and number of tumors, tumor capsule defect, histological differentiation, portal venous invasion and microvascular invasion (Table 2). Immunohistochemical staining showed that Hep Par 1 expression was negative and CK-19 expression was positive in all 131 cases (Figure 1).

Outcomes

No hospital death occurred in all the 131 ICC cases. The median postoperative survival of the ICC patients with positive AFP and with negative AFP was 37 mo and 28 mo respectively. The cumulative 1-year and 3-year survival rate of the positive AFP group was 68.7% and 46.8% respectively, both higher than 64.6% and 40.4% of the negative AFP group, though the difference was not statistically significant. Possible risk factors affecting survival included tumor size > 3 cm ($P = 0.014$), lymph node metastasis ($P < 0.0001$), portal venous invasion ($P = 0.006$), and the number of tumors ≥ 2 ($P < 0.0001$).

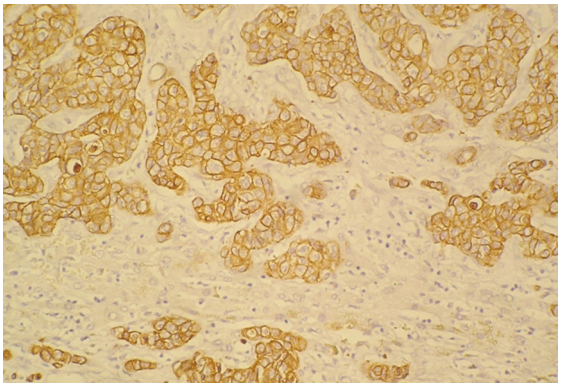

Figure 1 Representative sections showing immunohistochemical expression of CK-19 in intrahepatic cholangiocarcinoma (× 200).

DISCUSSION

Human AFP is a fetal glucoprotein with a molecular weight of about 72 kDa. Under physiological conditions, it is synthesized by fetal hepatocytes, yolk sac cells and gastrointestinal cells. AFP level begins to decrease gradually to < 10 ng/mL by 300 d of birth. Since detection of AFP in the serum of HCC patients in 1963, AFP has been widely used for screen examination and clinical diagnosis as an HCC tumor marker. In 60%-70% HCC patients, serum AFP is higher than the normal range[1,3,4]. In addition, increased AFP is also found in other pathological conditions such as hepatic cirrhosis, extensive hepatic necrosis, chronic hepatitis, pregnancy, gonadal fetal tumors and digestive tract tumors including gastric carcinoma, pancreatic carcinoma and gallbladder carcinoma. Positive AFP is rarely seen in ICC patients. A series of studies from a Japanese liver cancer research team showed that 19% ICC patients had a serum AFP level > 20 ng/mL, 10.3% > 200 ng/mL, and only 6.3% ICC patients had a serum AFP level > 1000 ng/mL[1]. In our series of 131 ICC patients, 32 patients (24.4%) had positive AFP, including 13 patients (9.9%) > 200 ng/mL, and 6 patients (4.5%) > 1000 ng/mL. The exact mechanism of how AFP is synthesized in ICC is not clear.

We found that ICC patients with positive AFP were associated with HBV infection and cirrhosis. This clinical feature is similar to that of HCC. What is consistent with ICC is that transaminase (a biomarker reflecting hepatic impairment) was higher in the positive AFP group than in the negative AFP group. Yamamoto *et al*[9] reported that ICC patients who were preoperatively diagnosed as having HCC had a relatively high rate of HCV infection. In ICC patients presenting with a high level of AFP and a low level of CA19-9, surgical treatment similar to HCC should be considered. Okuda *et al*[10] found that in ICC patients with positive Lens culinaris agglutinin-A-reactive AFP (AFP-L3), the hepatitis viruses infection rate was as high as 60%.

Lymph node metastasis is a common event in ICC; while it occurs rarely in HCC[11]. The data obtained from our study showed that the lymph node metastasis rate was low in ICC patients with positive AFP. What is consistent with previous studies is that lymph node metastasis is an important factor affecting the prognosis of ICC[12]. We found that the 1-year and 3-year survival rate of the positive AFP group was higher than that of the negative AFP group. This may be due to the lower lymph node metastasis rate of ICC patients with positive AFP. However, as the capacity of our cases is small and the follow-up period is not long enough, this statistical difference may not be significant.

The pathogenesis of ICC remains unclear. Recent studies show that HCC, ICC and many other tumors may originate from stem cells[13]. It is generally accepted that adult hepatic stem cells are hepatic oval cells. They are a group of intrahepatobiliary multi-potential differentiation cells, capable of differentiating to hepatobiliary cells and to hepatic cells. These cells are mainly located in the fetal liver or the hepatobiliary terminal Hering tube in adults. In normal physiological conditions, the number of oval cells is very small, and they are in a resting state. When the hepatic parenchyma is severely damaged, or regeneration of the hepatic cells is inhibited by virus, drugs, hepatic toxins or carcinogens, oval cells are activated, proliferating in large numbers and differentiating to hepatic and hepatobiliary cells to repair and reconstruct the liver[13]. AFP is not only an indicator of cell de-differentiation or immaturity but an important sign of hepatic stem cells[14]. Wang *et al*[15] reported that the expression rate of hepatic stem cell marker CK7 and CK19 was 100% in 12 ICC patients, while the expression rate of c-kit, Thy-1 and AFP was 41.7%, 33.3% and 33.3%, respectively. Transformation of oval cells to ICC cells was also observed. AFP synthesis in ICC suggests that ICC may originate from hepatic stem cells that underwent malignant transformation[16]. However, this presumption awaits verification by more studies.

Liver fluke infection (*Clonorchis sinensis or Opisthorchi viverrini*)[17,18], primary sclerosing cholangitis (PSC)[19,20], and hepatolithiasis are thought to be the risk factors for ICC[21,22]. Multiple studies in recent years show that viral hepatitis and hepatic cirrhosis are not only closely related to HCC but to ICC[23-26]. Our most recent case-control study showed that HBV infection is the possible pathogenic factor causing ICC in Chinese populations[27]. Proliferation of large numbers of oval cells was seen in chronic HBV and hepatic cirrhosis[28-30]. HBV infection may induce activation of oval cells, and this process may be accompanied with abnormal genetic alteration[31], which in turn triggers malignant transformation of oval cells.

In summary, ICC patients with positive AFP share many clinicopathologic similarities with HCC.

COMMENTS

Background

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic malignant tumor next to hepatocellular carcinoma (HCC). Serum a-fetoprotein (AFP), as a tumor marker of HCC, has been widely used in clinical practice. In about 19% ICC patients, serum AFP is also positive (> 20 ng/mL), but there is little knowledge about the clinicopathologic features of such patients.

Research frontiers

One hundred and thirty one patients who underwent surgical dissection for pathologically confirmed ICC were divided into a positive AFP (> 20 ng/mL) group (n = 32) and a negative AFP group (n = 99), whose clinicopathologic features were analyzed and compared.

Innovations and breakthroughs

The positive rate of HBsAg and liver cirrhosis of the positive AFP group was higher than that of the negative AFP group, while the positive rate of CA19-9 (> 37 U/mL) and the lymph node metastasis rate was lower.

Applications

AFP synthesis in ICC suggests that ICC may originate from hepatic stem cells that underwent malignant transformation[16]. However, this presumption awaits verification by more studies.

Peer review

This paper by Yan-Ming Zhou is an interesting study that describes the clinicopathologic characteristics of patients affected by intrahepatic cholangiocarcinoma with positive and negative serum AFP. This study includes 131 patients who underwent surgical dissection for pathologically confirmed ICC. The authors, concluding that ICC patients with positive AFP share many clinicopathologic similarities with HCC, suggest new perspectives in the management of intrahepatic cholangiocarcinoma.

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RAPID COMMUNICATION

Risk factors for alcohol-related liver injury in the island population of China: A population-based case-control study

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Abstract

AIM: To investigate the association of alcohol dose, duration of drinking and obesity with abnormal alcohol-related liver injury indicators, the prevalence of alcohol-related liver injury in the island population of China.

METHODS: Randomized multistage stratified cluster sampling from the island population of China was used in the population-based case-control study. Then interview, physical examination, laboratory assessments and ultrasonography were done.

RESULTS: Daily alcohol intake ⩾ 20 g, duration of drinking ⩾ 5 years and obesity were closely related to alcohol-related liver injury ($P < 0.05$). The odds-ratio (OR) (95% CI) was 1.965 (1.122-3.442), 3.412 (1.789-6.507) and 1.887 (1.261-2.824), respectively. The prevalence rate of alcohol-related liver injury in ⩾ 20 g daily alcohol intake group and < 20 g daily alcohol intake group was 37.14% and 12.06%, respectively. The prevalence rate of alcohol-related liver injury in ⩾ 5 years drinking group and < 5 years drinking group was 34.44% and 8.53%, respectively. No significant dose-response relation was found between daily alcohol intake and abnormal alcohol-related liver injury indicators as well as between duration of drinking and abnormal alcohol-related liver injury indicators. There was no significant difference in the prevalence of alcohol-related liver injury between beer drinking group and yellow rice wine drinking group, hard liquor drinking group, multiple drinking group.

CONCLUSION: The risk threshold of daily alcohol intake is 20 g and duration of drinking inducing alcohol-related liver injury 5 years in the island population of China. Liver injury induced by obesity should be concerned.

Key words: Alcohol; Liver injury; Prevalence; Case-control study; Epidemiology

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INTRODUCTION

Alcohol-induced liver disease remains one of the most common causes of chronic liver diseases[1]. Studies on alcoholic liver disease (ALD) have drawn wide attention in the Western world[2-5]. It was reported that ALD should be defined as an alcohol-associated lifestyle disease[6]. The predisposition to ALD is largely governed by gene-environment interactions. In recent years, along with the improved living standard and increased alcohol consumption, several epidemiological surveys showed that it has become a serious public health problem in China[7-10]. The island population in East China is a specific cluster of population. They feed themselves mainly on fishing, spend most of their time on sea-going ships, and consume a large amount of alcohol compared to the inland population. However, few population-based ALD studies are available from islands in China. Therefore, it is currently difficult to evaluate alcohol-related liver injury in the island population. Certainly, it is extremely important to select a sensitive and specific indicator in epidemiological survey. ALD is characterized by elevated serum gamma-glutamyltranspeptidase (GGT), aspartate aminotransferase

(AST) and alanine aminotransferase (ALT)[11-13]. According to the Practical Guidelines for Alcoholic Liver Disease published by the American College of Gastroenterology in 1998[14], GGT, AST and ALT were used as indicators of alcohol-related liver injury in this survey. We conducted a population-based case-control study to investigate the association of alcohol dose, duration of drinking and obesity with alcohol-related liver injury in East China.

MATERIALS AND METHODS

Study design and sample selection

We assigned a number to each of the counties is located along the coast of Zhejiang Province, and randomly selected one county (Xiangshan County). We randomly selected two islands (Hepu and Dongmen) of the 5 islands in the Xiangshan County and 9 villages of the 12 villages in the Hepu and Dongmen islands from August 2006 to September 2006. All individuals investigated in this study were at the age of over 18 years. All procedures were approved by the Ethics Committee of Zhejiang University School of Medicine. Each method and potential risks were explained in detail to the participants who gave their written informed consent before the survey.

Through a stratified multistage probability cluster sampling method, we acquired a representative sample from the island population in Zhejiang Province. We investigated 814 individuals aged 18 years or more in this survey, and obtained the complete data on 782 individuals. However, 129 individuals reporting clinical diagnosis of chronic viral hepatitis, schistosomiasis japonica (according to their epidemiological history, enzyme-linked immunosorbent assay results), cirrhosis (according to their medical history and ultrasonography results), or other severe diseases (mainly including drug-induced liver disease, cancer, pancreatitis, kidney disease, *etc,* based on their medical history) were excluded. Therefore, complete data were collected from 653 individuals. Their HBsAg and anti-HCV were negative. All individuals had no history of drug-induced liver disease and other severe diseases. There was no significant difference in the mean age between males and females. The mean age of males and females was 50.11 ± 13.48 years and 50.56 ± 11.72 years, respectively. There was also no significant difference in the mean BMI between males and females. The mean BMI of males and female was 24.60 ± 3.83 kg/m^2 and 24.54 ± 3.58 kg/m^2, respectively.

Interview

A face-to-face interview was conducted by trained physicians using a standardized questionnaire at the local community hospital. Data on demographic variables, alcohol drinking status, medical history and health behavior were collected from the questionnaire. Educational attainment of the followed up individuals was categorized into 5 groups according to the years of education (0, 1-6, 7-9, 10-12, ⩾ 12 years). Based on smoking habit grading[15], the followed up individuals were categorized into non-smoker group (never and cessation of smoking for more than 6 mo), smoking addict group (daily smoking for more than 6 mo), and smoking non-addict group (cessation of smoking or daily smoking for less than 6 mo). A series of questions of alcohol use included quantity of alcohol intake each time, times of alcohol intake each day, months of alcohol intake each year, years of alcohol intake, types and alcoholicity of alcoholic beverage, drinking and dietary habits. From the above data, we calculated the average daily alcohol intake (g), total alcohol intake (kg), and duration of drinking (years) by alcohol dose convert formula[16].

Physical examination

All followed up individuals were invited to have a physical examination at the local community hospital after the face-to-face interview. The followd up individuals were required to fast overnight. Body measurements were performed by a trained medical professional using a standardized protocol. Body weight and standing height were measured in light indoor clothing without shoes. Body mass index (BMI) was then calculated as mass (kg)/height (m)^2. The followd up individuals were divided into non-obese (BMI < 25 kg/m^2) group or obese (BMI ⩾ 25 kg/m^2) group as previously described[17]. Blood pressure was measured with an electronic blood pressure monitor (Omron HEM-746C, Omron Healthcare Inc., Bannockburn, Illinois, USA) on the right arm of the followd up individuals at a comfortable sitting position after a 5-min rest. Three measurements were taken. The second and third pressure readings were averaged and used for analysis. Diagnosis of hypertension was based on The JNC 7 Report[18] or on the current use of anti-hypertensive medications.

Laboratory assessments

Peripheral venous blood samples were collected after physical examination and centrifuged at 3000 r/min for 15 min at 4℃. After being frozen, the samples were shipped on dry ice to Department of Clinical Laboratory, First Affiliated Hospital, School of Medicine, Zhejiang University, and stored at -80℃. Blood samples were taken to check alcohol-related liver injury indicators which reflect the changes in the alcohol-related liver injury[14,16], including ALT, AST, GGT. Also, HBsAg, anti-HCV and enzyme-linked immunosorbent assay (ELISA) for schistosomiasis japonica were detected. All serum biochemistries were measured with a Hitachi 7600-110 automatic analyzer (Hitachi co., Tokyo, Japan). Reference value ranges of all indexes were based on the biochemistry criteria of Department of Clinical Laboratory, First Affiliated Hospital, School of Medicine, Zhejiang University. According to the Practical Guidelines for Alcoholic Liver Disease published by the American College of Gastroenterology in 1998[14], abnormal alcohol-related liver injury indicators were defined based upon AST > ALT (ALT or AST exceeding the upper normal level) or GGT exceeding the upper normal level.

Ultrasonographic examination

Hepatic ultrasonography for all individuals was performed by the same experienced ultrasonographist using a GE Logic Book XP portable ultrasound with a 3.5 MHz probe. Ultrasonographic diagnosis of cirrhosis followed the ultrasonographic criteria[19].

Table 1 Relationship between variables and alcohol-related liver injury detected by using univariate logistic-regression

Variable	β	S.E.	Wald χ^2	*P*	OR	95% CI
Male gender	1.152	0.228	25.448	0.000	3.163	2.022-4.948
Age	-0.008	0.007	1.162	0.281	0.992	0.978-1.006
Education level	0.061	0.127	0.229	0.632	1.063	0.829-1.362
Unmarried state	-0.144	0.408	0.125	0.724	0.866	0.389-1.926
Smoking	0.810	0.190	18.184	0.000	2.248	1.549-3.262
Daily alcohol intake ⩾ 20 g	1.460	0.201	52.562	0.000	4.307	2.902-6.392
Duration of drinking ⩾ 5 years	1.729	0.237	53.324	0.000	5.633	3.542-8.958
Total alcohol intake ⩾ 36.5 kg	1.506	0.208	52.582	0.000	4.507	3.000-6.711
Hypertension	0.473	0.209	5.128	0.024	1.605	1.066-2.418
Obesity	0.671	0.188	12.673	0.000	1.956	1.352-2.829

Table 2 Multivariate logistic-regression analysis of alcohol-related liver injury and selected variables

Variable	β	S.E.	Wald χ^2	*P*	OR	95% CI
Daily alcohol intake ⩾ 20 g	0.676	0.286	5.584	0.018	1.965	1.122-3.442
Duration of drinking ⩾ 5 years	1.227	0.329	13.884	0.000	3.412	1.789-6.507
Obesity	0.635	0.206	9.518	0.002	1.887	1.261-2.824

Table 3 Prevalence rate of alcohol-related liver injury (%)

Variable	Total ALI		ALI in obese		ALI in non-obese	
Daily alcohol intake(g)						
< 20	45/373	(12.06)	22/157	(14.01)	23/216	(10.65)
⩾ 20	104/280	(37.14)	60/119	(50.42)	44/161	(27.33)
Duration of drinking (yr)						
<5	25/293	(8.53)	12/118	(10.17)	13/175	(7.43)
⩾ 5	124/360	(34.44)	70/158	(44.30)	54/202	(26.73)

ALI: Alcohol-related liver injury.

Statistical analysis

We established a database using Epi Data 3.0 software (The EpiData Association, Odense, Denmark). Two typists recorded the data respectively and checked each other, corrected errors until two pieces of data were consistent. Statistical analysis was performed with SPSS 13.0 statistical package (SPSS Inc., Chicago, Illinois, USA). The mean value for different groups was compared using Student *t*-test. Chi-square (χ^2) test was used for comparing group ratios. We carried out univariate and multivariate stepwise logistic regression analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Risk factors for abnormal alcohol-related liver injury indicators

Of the 653 individuals, 149 were diagnosed having abnormal alcohol-related liver injury indicators in this study. Univariate logistic-regression analysis showed that male gender, smoking, ⩾ 20 g daily alcohol intake, ⩾ 5 years drinking, ⩾ 36.5 kg total alcohol intake, hypertension, obesity were closely related to abnormal alcohol-related liver injury indicators, while age, education level, unmarried state were not significantly related to abnormal alcohol-related liver injury indicators (Table 1).

Multivariate stepwise logistic-regression analysis showed that ⩾ 20 g daily alcohol intake, ⩾ 5 years drinking and obesity were closely related to abnormal alcohol-related liver injury indicators (Table 2). Compared to the < 20 g daily alcohol intake group, the odds-ratio (OR, 95% CI) of abnormal alcohol-related liver injury indicators in the ⩾ 20 g daily alcohol intake group was 1.965 (1.122-3.442). Compared to the < 5 years drinking group, the OR (95% CI) of abnormal alcohol-related liver injury indicators in the ⩾ 5 years drinking group was 3.412 (1.789-6.507).

Table 4 Relationship between different daily alcohol intake and BMI

Group	ALI	Normal	χ^2	*P*	OR	95%CI
X_0	23	193	-	-	-	-
X_1	44	117	17.564	0.000	3.156	1.813-5.492
X_2	22	135	0.970	0.325	1.367	0.732-2.553
X_3	60	59	65.121	0.000	8.534	4.864-14.972
Y_0	13	162	-	-	-	-
Y_1	54	148	23.911	0.000	4.547	2.385-8.668
Y_2	12	106	0.678	0.410	1.411	0.620-3.209
Y_3	70	88	60.338	0.000	9.913	5.194-18.918

X_0: Daily alcohol intake < 20 g and BMI < 25 kg/m^2; X_1: Daily alcohol intake ⩾ 20 g and BMI < 25 kg/m^2 group; X_2: Daily alcohol intake < 20 g and BMI ⩾ 25 kg/m^2; X_3: Daily alcohol intake ⩾ 20 g and BMI ⩾ 25 kg/m^2. Y_0: Drinking time < 5 years and BMI < 25 kg/m^2; Y_1: Drinking time ⩾ 5 years and BMI < 25 kg/m^2; Y_2: Drinking time < 5 years and BMI ⩾ 25 kg/m^2; Y_3: Drinking time ⩾ 5 years and BMI ⩾ 25 kg/m^2.

Prevalence of alcohol-related liver injury

Based on the daily alcohol intake and BMI, 216 subjects were assigned to control group (daily alcohol intake < 20 g and BMI < 25 kg/m^2), 161 to excessive drinking group (daily alcohol intake ⩾ 20 g and BMI < 25 kg/m^2), 157 to obese group (daily alcohol intake < 20 g and BMI ⩾ 25 kg/m^2), 119 to excessive drinking and obese group (daily alcohol intake ⩾ 20 g and BMI ⩾ 25 kg/m^2). The prevalence rate of abnormal alcohol-related liver injury indicators in the four groups was 10.7%, 27.3%, 14.0% and 50.4%, respectively (Table 3). Compared to the control group, the OR (95% CI) of abnormal alcohol-related liver injury indicators in the other groups was 3.156 (1.813-5.492, $P = 0.000$), 1.367 (0.732-2.553, $P = 0.325$), 8.534 (4.864-14.972, $P = 0.000$), respectively (Table 4).

Table 5 Dose-response to daily alcohol intake, drinking time and alcohol-related liver injury

Characteristics	Total	ALI	χ^2	*P*	OR (95%CI)	PR (%)	PR
Daily alcohol intake (g)							
< 20	373	45	-	-	-	12.06	1.00
20-40	76	25	20.817	0.000	3.573 (2.019-6.324)	32.89	2.73
40-80	73	27	28.011	0.000	4.278 (2.424-7.552)	36.99	3.07
80-160	65	24	25.774	0.000	4.267 (2.360-7.715)	36.92	3.06
⩾ 160	66	28	37.283	0.000	5.371 (3.010-9.584)	42.42	3.52
Duration of drinking (yr)							
< 5	293	25	-	-	-	8.53	1.00
5-10	22	4	2.279	0.131	2.382 (0.748-7.587)	18.18	2.13
10-20	58	22	36.082	0.000	6.551 (3.351-12.806)	37.93	4.45
20-40	206	76	60.265	0.000	6.267 (3.808-10.313)	36.89	4.32
⩾ 40	74	22	23.773	0.000	4.535 (2.379-8.647)	29.73	3.49

ALI: Alcohol-related liver injury; PR (%): Prevalence rate; PR: Prevalence ratio.

Based on the duration of drinking and BMI, 175 subjects were assigned to control group (duration of drinking < 5 years and BMI < 25 kg/m^2), 202 subjects to long-term drinking group (duration of drinking ⩾ 5 years and BMI < 25 kg/m^2), 118 to obese group (duration of drinking < 5 years and BMI ⩾ 25 kg/m^2), 158 to long-term drinking and obese group (duration of drinking ⩾ 5 years and BMI ⩾ 25 kg/m^2). The prevalence rate of abnormal alcohol-related liver injury indicators in the four groups was 7.4%, 26.7%, 10.2% and 44.3%, respectively (Table 3). Compared to the control group, the OR (95% CI) of abnormal alcohol-related liver injury indicators in the other groups was 4.547 (2.385-8.668, $P = 0.000$), 1.411 (0.620-3.209, $P = 0.410$), 9.913(5.194-18.918, $P = 0.000$), respectively (Table 4).

Dose-response relation of alcohol intake with alcohol-related liver injury

Compared to the < 20 g daily alcohol intake group, the OR of abnormal alcohol-related liver injury indicators in the other groups (daily alcohol intake was 20-40 g, 40-80 g, 80-160 g, ⩾ 160 g, respectively) was 3.573 ($P = 0.000$), 4.278 ($P = 0.000$), 4.267 ($P = 0.000$), 5.371 ($P = 0.000$), respectively (Table 5). The prevalence rate of abnormal alcohol-related liver injury indicators in the other groups was 12.1%, 32.9%, 37.0%, 36.9%, 42.4%, respectively. Compared to the < 20 g daily alcohol intake group, the prevalence rate in the other groups was 2.73, 3.07, 3.06 and 3.52, respectively. Compared to the < 5 years drinking group, the OR of abnormal alcohol-related liver injury indicators in the other groups (drinking duration was 5-10 years, 10-20 years, 20-40 years, ⩾ 40 years) was 2.382 ($P = 0.131$), 6.551 ($P = 0.000$), 6.267 ($P = 0.000$), 4.535 ($P = 0.000$), respectively (Table 5). The prevalence rate of abnormal alcohol-related liver injury indicators in the other groups was 8.5%, 18.2%, 37.9%, 36.9%, 29.7%, respectively. Compared to the < 5 years drinking group, the prevalence ratio in the other groups was 2.13, 4.45, 4.32 and 3.49, respectively.

Different types of alcoholic beverage and alcohol-related liver injury

Of the 313 drinkers, 126 were beer drinkers, 36 yellow rice wine drinkers, 41 hard liquor drinkers and 110 multiple drinkers. The prevalence rate of abnormal alcohol-related liver injury indicators in these four kinds of drinkers was 30.2%%, 38.9%, 39.0% and 40.0%, respectively. Compared to the beer drinkers, no significant difference was found in the prevalence rate of abnormal alcohol-related liver injury indicators among the other groups (Table 6).

Table 6 Different types of alcoholic beverage and alcohol-related liver injury

Type of beverage	Total	ALI (*n*, %)	Daily intake (g)	χ^2	*P*
Beer	126	38 (30.16)	40.26 ± 31.35	-	-
Yellow rice wine	36	14 (38.89)	78.15 ± 57.88	0.979	0.322
Hard liquor	41	16 (39.02)	107.09 ± 84.38	1.111	0.292
Multiple	110	44 (40.00)	167.26 ± 109.60	2.509	0.113
Total	313	112 (35.78)			

ALI: Alcohol-related liver injury.

Analysis of obesity

Obesity (BMI ⩾ 25 kg/m^2) was also found to be an important risk factor for liver injury (Tables 1 and 2). Multivariate stepwise logistic-regression analysis showed that the OR (95% CI) of abnormal alcohol-related liver injury indicators was 1.887 (1.261-2.824) in the BMI ⩾ 25 kg/m^2 group compared to the BMI < 25 kg/m^2 group (Table2).

DISCUSSION

Liver is the major alcohol processing organ. Chronic heavy drinking induces liver injury and results in alcoholic liver disease, even irreversible alcoholic liver cirrhosis[20]. Since ALD has no specific clinical features[21] and no specific laboratory tests[14] are available for it, its diagnosis is currently based on drinking history, related laboratory assessments and imaging[16,22,23]. Certainly, liver biopsy is the gold standard for diagnosis of ALD[24], but it is hard to use this invasive examination in population-based epidemiological surveys. Irie *et al*[11] found that GGT synthesis and protein expression are increased in ALD, leading to elevated serum levels of GGT

that are commonly noted in patients with the disease. Elevated GGT is somewhat more sensitive at 69%-73% with a specificity of 65%-80% for excessive alcohol consumption[25,26]. An elevated serum AST in relation to serum ALT has been proposed as an indicator of alcohol-induced organ damage[27]. It was reported that most patients with high alcohol consumption but without severe liver disease do not have an AST/ALT ratio above one. A high AST/ALT ratio suggests advanced alcoholic liver disease[13]. Therefore, we chose the GGT and AST/ALT ratio as the indicators of alcohol-related liver injury in this epidemiological survey.

In the present study, logistic-regression analysis demonstrated that daily alcohol intake ⩾ 20 g, drinking time ⩾ 5 years and obesity were important risks for abnormal alcohol-related liver injury indicators. The risk for abnormal alcohol-related liver injury indicators was 1.965-fold higher in the ⩾ 20 g daily alcohol intake group than in the < 20 g daily alcohol intake group, while the risk for abnormal alcohol-related liver injury indicators was 3.412-fold higher in the ⩾ 5 years drinking group than in the < 5 years drinking group. However, the risk thresholds of alcohol intake-induced alcoholic liver injury were different in different areas. No uniformed conclusion has been achieved in alcohol intake- induced alcoholic liver injury[28-32]. It was reported that even low alcohol level is a significant risk of developing liver disease[33]. Therefore, significant differences exist among different racial and ethnic groups and even in different individuals[34,35]. It has been shown that genotype of ethanol metabolizing enzyme genes in the Chinese population is different from Western population[36]. Therefore, genetic factors in the island population from East China need to be further studied.

As to the dose-response relation of alcohol intake and abnormal alcohol-related liver injury indicators, our results demonstrate that there was no significant dose-response relation between daily alcohol intake, drinking time and abnormal alcohol-related liver injury indicators. Kamper-Jorgensen *et al*[37] showed that alcoholic threshold has a greater effect on the mortality of alcoholic cirrhosis.

Obesity is also an important risk factor for liver injury. In this study, logistic-regression analysis showed that the risk of obesity for abnormal alcohol-related liver injury indicators was 1.887. Some studies found that obesity is an independent risk factor for liver injury in alcohol drinkers[38-40]. In our study, the prevalence rate of alcohol-related liver injury in the non-obese group, non-excessive drinking or no long-term drinking group was lower than that in the obese group, excessive drinking group or long-term drinking group. The highest prevalence rate of alcohol-related liver injury was found in the obese and excessive drinking/long-term drinking group. There was a difference in the odds-ratio of abnormal alcohol-related liver injury indicators between the non-obese and excessive drinking/long-term drinking group, the obese and excessive drinking/long-term drinking group and control group ($P < 0.05$), while was no difference in the odds-ratio of abnormal alcohol-related liver injury indicators between the obese and non-excessive drinking group and no long-term drinking group, suggesting that the specificity of GGT and AST/ALT ratio can be sued as the indicators of alcohol-related liver injury.

However, hepatotoxic consequences of obesity and ethanol ingestion have important prognostic implications and might be useful to formulate body mass index-based guidelines for "safe" alcohol consumption[41].

No significant difference was found in the morbidity of abnormal alcohol-related liver injury indicators between the beer and other groups, suggesting that the types of alcoholic beverage are not closely related with abnormal alcohol-related liver injury indicators. Therefore, we believe that alcohol intake plays a more significant role in liver injury than the type of alcoholic beverage.

The island population from East China is a specific cluster of population. They feed themselves mainly on fishing and spend most of their time on sea-going ships. Although they consume a large amount of alcohol every day, their alcohol-related liver injury is not very severe. Most individuals in this area are alcohol drinkers. It was reported that treatment modalities aiming at reducing alcohol intake in alcohol-dependent patients include psychological, pharmacological and psychological therapies, but many patients benefit more from pharmacological therapy[42]. We believe that epidemiology study of the island population from East China is more important than that of the inland population. Certainly, it is more useful to analyze the differences in island and inland populations, including drinking habit, diet habit, living and working pressure, genotype, *etc*. ALD is governed by gene, environmental and psychological factors. We will continue to pay close attention to the island population from East China.

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COMMENTS

Background

The island population from East China is a specific cluster of population. They consume a large amount of alcohol compared to the inland population. However, few population-based studies on alcoholic liver disease (ALD) are available from islands in China. We conducted a population-based case-control study to investigate the association of alcohol consumption, drinking time and obesity with liver injury in the island population from East China.

Research frontiers

The association of alcohol consumption and drinking time with alcohol-related liver injury in the island population from East China was studied.

Innovations and breakthroughs

The risk threshold of daily alcohol intake is 20 g and the drinking time that induces alcohol-related liver injury is 5 years in the island population from East China. Obesity-induced liver injury should also be concerned. Whether hepatotoxic consequences of obesity and alcohol ingestion are additive or synergistic is worthy to be further studied.

Applications

The results are useful to analyze the differences in the island and inland

population, including drinking habit, diet habit, living and working pressure, genotype.

Terminology

Gamma-glutamyltranspeptidase (GGT) and aspartate aminotransferase/ alanine aminotransferase (AST/ALT) ratio are the characteristics of alcoholic liver disease (ALD). Abnormal alcohol-related liver injury indicators are defined based upon the condition that aspartate aminotransferase / alanine aminotransferase (AST>ALT) (ALT or AST exceeding the upper normal level) or GGT exceeding the upper normal level.

Peer review

The paper describes the association of alcohol consumption and drinking time with alcohol-related liver injury in the island population from East China. The topic is highly interesting. The island population should be concerned in the follow-up research in future.

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RAPID COMMUNICATION

Endoscopic diagnosis of gastrointestinal graft-versus-host disease

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Abstract

AIM: To evaluate the diagnostic value of endoscopy in patients with gastrointestinal graft-versus-host disease (GI GVHD).

METHODS: We identified 8 patients with GI GVHD following allogeneic hematopoietic stem cell transplantation (HSCT). GVHD was defined histologically as the presence of gland apoptosis, not explained by other inflammatory or infectious etiologies.

RESULTS: The symptoms of GI GVHD included anorexia, nausea, vomiting, watery diarrhea, abdominal pain, GI bleeding, *etc*. Upper endoscopic appearance varied from subtle mucosal edema, hyperemia, erythema to obvious erosion. Colonoscopic examination showed diffuse edema, hyperemia, patchy erosion, scattered ulcer, sloughing and active bleeding. Histological changes in GI GVHD included apoptosis of crypt epithelial cells, dropout of crypts, and lymphocytic infiltration in epithelium and lamina propria. The involvement of stomach and rectocolon varied from diffuse to focal.

CONCLUSION: Endoscopy may play a significant role in early diagnosis of GI GVHD patients following allogeneic HSCT, and histologic examination of gastrointestinal biopsies is needed to confirm the final diagnosis.

Key words: Gastrointestinal graft-versus-host disease; Endoscopy; Diagnosis; Allogeneic hematopoietic stem cell transplantation

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INTRODUCTION

Allogenic hematopoietic stem cell transplantation (HSCT) is increasingly performed for a variety of disorders, including acute and chronic leukemia, hematologic malignancies, and marrow failure states[1]. Graft-versus-host disease (GVHD) is the leading cause of morbidity and mortality after allogeneic HSCT[2,3]. Gastrointestinal (GI) complaints are relatively common within the first 100 d following allogeneic HSCT[4,5]. Although nausea, vomiting, anorexia and high-volume diarrhea are the common manifestations of GI GVHD, they may also be attributable to chemoradiation toxicity, medication side effects, or a variety of bacterial, fungal, viral infections. Thus, it is very difficult to establish the diagnosis of GI GVHD based on the clinical grounds alone[6].

Endoscopy with biopsy has been shown to be accurate in the identification of GVHD. Although previous reports have documented a high yield for rectal biopsy[7,8], upper GI biopsies are superior to rectal or rectosigmoid biopsies in the diagnosis of GVHD[9,10]. Thus further evaluation may be needed to establish the best diagnostic approach to GI GVHD.

Our aim in the present study was to demonstrate the endoscopic and histological features of GI GVHD. Eight patients with proven GI GVHD were included in the study, and we intended to evaluate the significance of endoscopy and biopsy in the diagnosis of GI-GVHD.

MATERIALS AND METHODS

Patients

From January 2002 to December 2006, eight patients with suspected GI GVHD 20 d following allogenetic HSCT at the First Affiliated Hospital of Soochow University were enrolled in this study. All patients were interviewed and the following data were recorded: age, gender, underlying disease and transplantation type, stool per day, stool volume, nausea, vomiting, diarrhea, anorexia, gastrointestinal bleeding and skin rash. Laboratory studies including liver chemistry tests were also recorded. Routine

Table 1 Characteristics of patients with GVHD

Case No./Sex/Age	Diagnosis	Conditioning regimens	Donor HLA match	GVHD prophylaxis	Stage grading
1/F/29	AML-M2	BU/CY	HLA 2-locus mismatched unrelated donor	CSA, ATG, MMFMTX	aGVHD grade Ⅳ
2/M/47	CML-CR	Fludarabine, Bu, ATG	HLA-identical sibling donor	CSA, MTX	cGVHD Limited
3/M/39	CML -CR	BU/CY	HLA-identical sibling donor	CSA, MTX	cGVHD/Limited
4/M/23	CML-CR	BU/CY	HLA-identical sibling donor	CSA, MTX	aGVHD grade Ⅳ
5/M/63	CML-CR	Fludarabine, Bu, ATG	HLA-identical sibling donor	CSA, MTX	aGVHD grade Ⅳ
6/F/35	ALL-CR	Me-CCNU, TBI, Ara-C, CY	HLA 2-locus mismatched related donor	CSA, MTX, MMF, ATG	aGVHD grade Ⅳ
7/M/42	ALL-CR	BU/CY, Me-CCNU, Ara-C	HLA 2-locus mismatched related donor	CSA, MTX, MMF, ATG	aGVHD grade Ⅲ
8/F/23	ALL-CR	Me-CCNU, TBI, Ara-C, CY	HLA 2-locus mismatched related donor	CSA, MTX, MMF, ATG Anti-CD25	aGVHD grade Ⅲ

HLA: Human leucocyte antigen.

stool examination and bacterial culture were performed for all patients. Cytomegalovirus (CMV) antigenemia was monitored twice weekly after conditioning regimens.

Histocompatibility and stem cell source

One patient with acute myeloid leukemia (AML) underwent 2-locus mismatched unrelated donor transplant. Four patients with chronic myeloid leukemia (CML) were recipients of a matched related donor (MRD) transplant. Three patients with acute lymphoblastic leukemia (ALL) received haploid related donor transplant. Peripheral blood hematopoietic stem cells were collected from donors in all but one case. One case with AML had hematopoietic stem cells harvested from bone marrow through Taiwan Marrow Donor Registry.

Conditioning regimen and GVHD prophylaxis

Following conditioning regimens were used: BuCy (busulfan 4 mg/kg per day for 4 d and cyclophosphamide 60 mg/kg per day for 2 d) for standard transplantation in patients 1, 3 and 4; Bu-Fludara-ATG (antihuman thymocyte globulin) (busulfan 4 mg/kg per day for 2 d, fludarabine 30 mg/m^2 per day for 6 d, and antithymocyte globulin 2.5 mg/kg per day for 4 d) for non-myeloablative transplantation in patients 2, 5; Me-CCNU (semustine)-TBI (total body radiation)-Cy (Me-CCNU 250 mg/m^2, -d_8; TBI 8Gy, -d_7; Ara-C (arabinosylcytosin) 4 g/m^2, -d_6, -d_5; Cy 1.8 g/m^2, -d_4, -d_3) for all ALL patients.

All patients received cyclosporine A (CSA) with short-course MTX (methotrexate) for the prophylaxis of GVHD. For patient 1 who underwent 2-locus mismatched unrelated transplantation, ATG and MMF (mycophenolic mofetil) were added. Treatment for patients with ALL was intensified and prolonged by using the combination of cyclosporine A, MMF, ATG and anti-CD25 antibody for GVHD prophylaxis.

Diagnostic criteria and GVHD grading system

GI GVHD could be diagnosed according to its clinical manifestations, endoscopic appearance and histopathological evaluation. Medication-induced side effects, chemoradiation toxicity or GI infections must be excluded. Specific histological criteria could establish the diagnosis of GI GVHD. Focal dropout and apoptosis of GI crypt epithelial cells are usually regarded as golden standard to diagnose GVHD. Acute GVHD (aGVHD) is defined as occurring within 20 to 100 d after transplantation and chronic GVHD (cGVHD) occurring 100 d after transplantation[5]. A clinical grading system based upon the degree of involvement for each of the organ systems was originally developed by investigators in Seattle[2]: (1) grade Ⅰ: 500-1000 cc stool/d, accompanied with anorexia and vomiting; (2) grade Ⅱ: 1000-1500 cc stool/d, histologically proven GVHD by endoscopic biopsies; (3) grade Ⅲ: 1500-2000 cc stool/d; (4) grade Ⅳ: over 2000 cc stool/d, accompanied with ileus and severe abdominal pain.

Gastrointestinal endoscopy and biopsy

If patients had persistent unexplained GI symptoms (diarrhea, nausea, vomiting, anorexia, abdominal pain or gastrointestinal bleeding) after transplantation, then upper endoscopy and/or colonoscopy were performed. Upper endoscopy with gastric biopsies of both antrum and body were performed in one patient, colonoscopy was performed with multiple biopsies of the ileum, right colon and rectosigmoid colon in 6 patients. A combination of upper endoscopy with colonoscopy and multiple biopsies was performed in another patient. For each patient, biopsies were systematically performed in the GI tract, two of which were transmitted to the microbiology department and studied further for bacterial, viral, or fungal pathogens. Another two biopsy specimens were immediately snap-frozen in liquid nitrogen and used for CMV immunohistochemical study. The remaining two biopsy specimens were fixed in formaldehyde, and further processed for paraffin embedding. Paraffin blocks were sectioned at 4 μm and stained with hematoxylin and eosin for routine histopathological examination.

RESULTS

Clinical presentation of GVHD

Of the eight patients, two developed grade Ⅲ acute GI GVHD, and four grade Ⅳ acute GI GVHD, the remaining suffered from limited chronic GI GVHD. Detailed data are listed in Table 1. The clinical manifestations of upper GI GVHD included nausea, vomiting, anorexia, and abdominal pain. Lower GI symptoms manifested as voluminous secretory diarrhea accompanied with abdominal bloating or pain. Three patients had intestinal bleeding, and only one patient had gastric bleeding (Table 2).

Table 2 Gastrointestinal symptoms of patients with GI GVHD

Case No. /Sex/Age	Nausea	Vomiting	Anorexia	Abdominal pain	Diarrhea	Gastrointestinal bleeding
1/F/29	+	+	+	+	+	+
2/M/47	+	+	+	-	-	-
3/M/39	-	-	-	-	+	+
4/M/23	+	+	-	+	+	+
5/M/63	-	-	-	+	+	-
6/F/35	+	-	-	+	+	+
7/M/42	+	-	+	+	+	-
8/F/23	+	+	-	-	+	-

Endoscopic findings

The endoscopic findings varied greatly. The first endoscopy for patient 1 with grade Ⅳ acute GVHD showed diffuse erythema with mucosal ozzing in the antrum and body of the stomach (Figure 1A). Because nausea, vomiting, melena and hematemesis persisted despite empiric treatment, emergency upper endoscopy and biopsy were repeated 1 wk later. The endoscopic appearance revealed a pale mucosal surface with reticulated submucosal small vessels accompanied with erosion and erythema in the antrum (Figure 1B). For the same patient, colonoscopy was performed after gastric bleeding was controlled, and disclosed extensive mucosal hyperemia and edema in the colon. In patient 2 with chronic GI GVHD, the upper endoscopic examination showed subtle mucosal edema with erythema in the antrum, but the appearance of the esophagus and duodenum was grossly normal. In patient 3 with chronic GVHD five months after transplantation, colonoscopic examination disclosed hemorrhagic spots, patchy erosions, and active bleeding. Patients 4, 5 and 6 showed similar diffuse damages, namely widespread erythema, multiple erosions and small ulcer. Two of the three patients had active bleeding in the colon (Figure 2A). Hemorrhagic spots and multiple shallow ulcers could be detected on the surface of rectocolon (Figure 2B). Patients 7 and 8 demonstrated widespread edema, erythema with multiple erosions without active bleeding in the total rectocolon.

Pathologic findings

In patient 1, histologic examination of gastric biopsy specimens showed focal dropout of crypt epithelial cells, variable lymphocytic infiltration of the epithelium and lamina propria, and colonic biopsies showed nonspecific inflammation. Gastric biopsies disclosed a crypt with multiple apoptotic cells in patient 2. Extensive mucosal erosions, shallow ulcer, sloughing and apoptosis of epithelial cells were found in patient 3. Extensive colonic mucosal erosion and necrosis were observed in patients 4 and 5, and biopsies of the colon in these patients showed clear histological evidence of acute GVHD. Biopsy specimens from patients 6, 7 and 8 illustrated numerous apoptotic bodies in crypts, and small lymphocytic infiltration of the adjacent lamina propria. CMV infection was not confirmed on biopsy specimens from seven patients by immunohistochemical study except for one patient with HLA 2-locus mismatched, in which colonic mucosa was weakly positive, but late antigen was negative

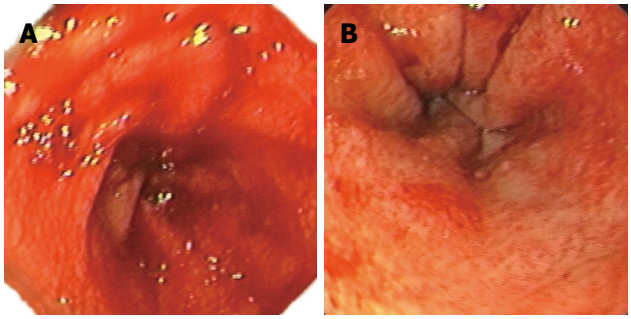

Figure 1 Upper endoscopy showing diffuse and active bleeding in the antrum and body of stomach 160 d after allo-BMT (**A**) and reticulated submucosal small vessels accompanied with erosion and erythema in the antrum 175 d after allo-BMT (**B**) in patient 1 with AML.

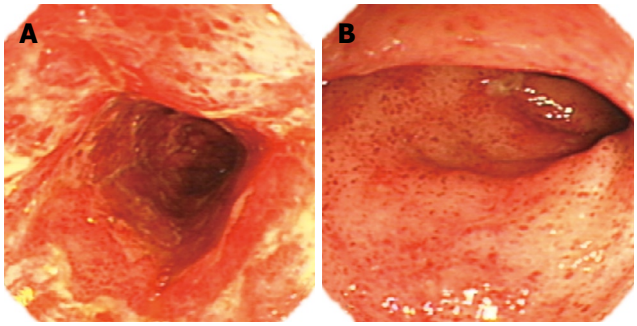

Figure 2 Colonoscopy disclosing mucosal erythema, severe erosions, multiple ozzing and sloughing in ascending colon (**A**) and extensive hemorrhagic spots, patchy erosions, and focal shallow ulcers in the rectum (**B**) in patient 4 with CML 90 d after allo-HSCT.

205 d after transplantation. Because this patient had concomitantly severe GI GVHD and skin involvement 24 d after allogeneic bone marrow transplantation (BMT), GI GVHD coexisted with CMV infection.

DISCUSSION

GVHD is the leading cause of morbidity and mortality after allogenic HSCT, occurring in up to 75% of patients[11]. According to the degree of involvement in each of the organ systems, acute GVHD can be clinically classified as grades Ⅰ-Ⅳ. High risk factors include HLA disparity, unrelated-donor transplantation, donor-recipient gender difference, old age, and infection[12]. In the present study, one young female patient who underwent two-locus HLA-mismatched unrelated BMT suffered from grade Ⅳ acute GI GVHD 24 d after transplantation.

The principal organs with involvement of acute GI GVHD include stomach, small intestine, and rectocolon[13], but esophageal acute GVHD is uncommon[14,15]. Roy *et al*[10] found that GVHD limited to the upper GI tract accounts for 18% of patients, GVHD involving the lower and upper GI tract accounts for 10%, and 26% of patients. The most prominent symptoms of GVHD involving the upper GI tract are anorexia, dyspepsia, nausea, vomiting, and, occasionally, abdominal pain[16]. Lower GI GVHD manifests as voluminous watery diarrhea (typically secretory in nature) accompanied with abdominal bloating,

ileus, and occasionally overt intestinal bleeding[17,18]. In contrast to acute GI GVHD, chronic GI GVHD differs markedly in distribution and histopathology. Esophageal involvement of chronic GI GVHD is not uncommon, but the stomach and intestine are rarely involved[19].

In the present study, colonoscopy disclosed scattered hemorrhagic spots and mucosal erosion in one patient with chronic GVHD.

Obviously, clinical manifestations of GI GVHD are nonspecific. There is a wide overlap of symptoms with many GI diseases. Toxicity from the regimen of cytoreductive therapy given before transplantation can cause symptoms of anorexia, nausea, vomiting, all of which are also characteristic of GVHD[2,20]. For most conditioning regimens, this variable is less important 20 d after transplantation, when toxicity to intestinal mucosa has largely resolved. A variety of bacterial, fungal and viral infections may affect the diagnosis of GI GVHD. Clinical manifestations of intestinal bacterial infection are mainly bloody stool and pathogenic bacteria can be confirmed from excreta. Endoscopy can also disclose mucosal erosion and pus moss. Fungal infections of the GI tract have become unusual since the routine use of prophylactic fluconazole, and fungas can be identified by examining stool specimens. In addition, since clinical symptoms of enteric CMV infection can resemble GVHD, all patients must undergo viral surveillance. Histologic identification of CMV infection is less sensitive than viral culture. Therefore, viral immunohistology and culture should be done if the patient is at a high risk for CMV infection. For more sensitive detection of CMV reactivation, polymerase chain reaction is also recommended[21,22].

As stated previously, patients with and without GI GVHD cannot be distinguished based entirely upon clinical findings. Accurate and timely diagnosis is essential, as early recognition and intervention may significantly improve the outcome[23,24]. Endoscopy combined with tissue biopsy is usually required to establish the diagnosis of acute GI GVHD. In a retrospective study, Terdiman and colleague[25] confirmed that acute upper GI GVHD is sensitive to many drugs if early diagnosis could be properly made. While treatment fails, upper GI GVHD may progress to lower GI. Therefore, upper GI GVHD is an early event. Our study revealed that upper endoscopic appearance of GVHD ranged from normal mucosa to erythema, erosion, ulceration and active bleeding. Normal endoscopic examinations have been reported in up to 21% of patients with histologically confirmed acute GVHD[20]. Sloughing of the mucosa is uncommon but high specific[26]. It is noted that discordance may be seen in different regions of the gut. In the present study, mucosal lesions in the antrum and body were more severe than those in the funds and duodenum, whereas the esophagus was less involved.

Enteric acute GVHD exhibits diffuse hyperemia, edema, erosion, and slough of mucosa, which can resemble severe ulcerative colitis[27]. In the present study, the grossly visible mucosal damage was uneven in distribution, sometimes appearing severely abnormal in one area while being unremarkable at other locations.

Since endoscopic appearance of GVHD is also nonspecific, endoscopic diagnosis cannot replace histopathological examinations. At present, endoscopy with biopsies remains the gold standard for the diagnosis of acute GI GVHD[2]. Histological criteria for GVHD are the presence of epithelial single-cell apoptosis and crypt cell dropout[28]. However, the reported mucosal site with the highest diagnostic yield (upper and/or lower) varies in studies. In a prospective study of HSCT patients with diarrhea and upper GI symptoms, Cox and his companies[20] discovered that the positive rate of gastric mucosal biopsies was 85% in 29 GVHD patients who were confirmed by histopathology and 58% in biopsies from duodenum and rectum-sigmoid colon. In another prospective study of 24 patients undergoing both upper and lower endoscopy[23], biopsies were obtained from the stomach, duodenum, ileum, right and rectosigmoid colon, while the biopsy site with the highest yield was the distal colon (82%), and a combination of upper endoscopy with sigmoidoscopy and colonscopy with ileal biopsies was equivalent (94%), suggesting that multiple biopsies should be obtained from stomach, duodenum, and rectum-sigmoid colon, in order to improve the accuracy and sensitivity of diagnosis. Many factors (chemoradiation toxicity, medication side effects, particularly CMV infection), can interfere with the histologic interpretation. Proton pump inhibitor (PPI) therapy is associated with increased apoptosis in antral biopsies. Biopsy from gastric fundus rather than from antrum may be preferable for the diagnosis of upper GI GVHD[3]. It is, therefore, important to rule out these factors in making a histologic diagnosis of GVHD after transplantation.

There is a discrepancy between endoscopic and histologic assessments of the severity of the disease[29]. Mucosal edema and erythema that are endoscopically impressive will be subtle when corresponding biopsies are assessed microscopically. In contrast, normal mucosa may display focal crypt epithelial apoptosis characteristic of GVHD. Thus, the correlation between endoscopic and histologic findings requires further investigation.

A clinical grading system based on the degree of lower GI symptoms (diarrhea volume, *etc*) does not consider the upper GI symptoms and endoscopic findings. Thus, an alternative, revised grading system needs to be proposed that takes into account the upper GI symptoms and endoscopic findings.

Roy *et al*[7] showed upper GI involvement is more common than lower GI in patients with GVHD confirmed by skin biopsy. Weisdorf *et al*[30] also confirmed that 59.7% of patients with GI GVHD have skin GVHD. Therefore, endoscopy with tissue biopsies may acquire positive results in patients with negative skin biopsies. It is noted that GI GVHD is not correlated with hepatic venous occlusion diseases (VOD).

It was reported that endoscopic examination is usually safe for patients with GVHD or occasional intestinal perforation, and oozing at the biopsy site due to thrombocytopenia[27]. Thus, a platelet count of more than 50×10^9/L is needed before endoscopic examination.

Because of the lack of sufficient samples, diagnostic endoscopic findings need further evaluation. In addition, endoscopists should cooperate with specialists in bone

marrow transplantation to standardize the biopsy location and the number of specimens, method and time to undertake gastroscopy and/or colonoscopy[23].

In summary, endoscopic findings are highly variable in diagnosis of GI GVHD. There is a discrepancy between endoscopic and histologic assessments of the severity of GI GVHD. Gastrointestinal biopsies are needed to confirm the diagnosis of GI GVHD.

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COMMENTS

Background

Allogenic hematopoietic stem cell transplantation (HSCT) is increasingly performed for a variety of disorders, such as acute and chronic leukemia, but many patients undergoing HSCT develop acute graft-versus-host disease (GVHD). GVHD involving the gastrointestinal (GI) tract is common, but it is difficult to establish the diagnosis of GI GVHD because of the nonspecific GI symptoms. Recognition of GI GVHD is critical for directing its specific therapy.

Research frontiers

The diagnosis of GI GVHD often depends on an endoscopic evaluation. The endoscopic appearance of GI GVHD can range from normal to mild edema or erythema to dramatic mucosal slough, but the mucosal damage caused by chemoradiation toxicity, side effects of medications, and enteric infections with viruses, bacteria, and fungi may occur. Although endoscopy with biopsy is commonly used in the evaluation of suspected GI GVHD, the best diagnostic approach remains undefined.

Innovations and breakthroughs

There is no standardized protocol for upper or lower endoscopy, biopsy number and location. This study demonstrated that endoscopic examinations and histologic evaluation of biopsies could be used to diagnose GI GVHD. There is a discrepancy between endoscopic and histologic assessments of the severity of GI GVHD.

Applications

The present study further demonstrated the endoscopic role in diagnosing GI GVHD in patients following allogeneic HSCT, and histologic examination of GI biopsies is needed to confirm the final diagnosis.

Terminology

GVHD: a condition that occurs following bone marrow transplantation or peripheral blood stem cell transplantation, in which lymphocytes from the graft attack specific tissues in the host. The skin, gut, and liver are the most severely affected. Drugs that suppress the immune reaction, such as steroids and cyclosporin A, reduce the severity of rejection.

Peer review

The present study reported eight patients with proven GI GVHD and demonstrated the role of endoscopic examinations and histologic evaluation of biopsies in diagnosing GI GVHD, which is very important in clinical practice.

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CASE REPORT

Extraintestinal heterotopic gastric tissue simulating acute appendicitis

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Abstract

We describe the case of a 68-year-old otherwise healthy male who presented to our emergency room with signs and symptoms of acute appendicitis. Exploratory surgery revealed a normal appendix. Further examination revealed an enlarged lymph node-like mass of tissue near the appendix, in the ileocecal mesentery. This mass was removed and was found to be inflamed heterotopic gastric tissue. Although reports of heterotopic gastric tissue in the literature are common, we believe that this case represents the first report of inflamed heterotopic gastric tissue simulating appendicitis.

Key words: Heterotopic gastric mucosa; Acute appendicitis

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Bender E, Schmidt SP. Extraintestinal heterotopic gastric tissue simulating acute appendicitis. *World J Gastroenterol* 2008; 14(14): 2268-2269 Available from: URL: http://www.wjgnet.com/1007-9327/14/2268.asp DOI: http://dx.doi.org/10.3748/wjg.14.2268

INTRODUCTION

Reports of the occurrence of heterotopic gastric tissue in the medical literature are common. Von Radhen *et al*[1] and Steele *et al*[2] have recently published case reports that included extensive reviews of the literature on this topic. Heterotopic gastric mucosa has been described in the esophagus and in the oral cavity, as a polyp in the distal ileum, as a mass in the rectum and anus, in the scrotum, in the hepatobiliary system, including the gallbladder and common bile duct, in the mediastinum and in the spinal cord. Typically heterotopic gastric tissue is asymptomatic. In contrast, we present a report of gastric heterotopia in a patient who presented at our institution with signs and symptoms of acute appendicitis. To our knowledge, this presentation of heterotopic gastric mucosa is unique to the medical literature.

CASE REPORT

The patient was a previously healthy 68-year-old male whose past medical history was significant only for benign prostatic hypertrophy treated with Cardura. He presented to the emergency room with approximately 6 h of right lower quadrant pain and nausea. The pain had started in the peri-umbilical area and had localized to the right lower quadrant by the time of his presentation. The patient denied having had this type of pain previously. On examination, the patient was tender in the right lower quadrant with voluntary guarding and a positive obturator sign. His white blood cell count was 10 000 with 89% granulocytes. His abdominal films showed a mild ileus pattern with no evidence of obstruction or free air.

The patient was taken to the operative suite and explored through a McBurney incision. A small amount of straw colored ascites was found upon entrance to the abdomen. The appendix was exceptionally long. However, it did not show any signs of inflammation. However, near the appendix, in the ileocecal mesentery, there was what appeared to be an enlarged, inflamed lymph node. This tissue was removed and sent to pathology for permanent stains. There was no evidence of a Meckel's diverticulum upon examination of the distal two feet of ileum. The right colon was otherwise normal in appearance and by palpation.

The tissue that had been thought to be an enlarged, inflamed lymph node was removed and established by pathology to be gastric heterotopic tissue with evidence of inflammation/gastritis (Figure 1).

Postoperatively, the patient's pain resolved. He was maintained on Prevacid (30 mg once daily) due to the concern that he might have additional areas of gastric heterotopic tissue. However, a technetium-99 scan did not show any areas of uptake postoperatively and he remains asymptomatic three years postoperatively.

DISCUSSION

We are presenting what we believe to be the first case of

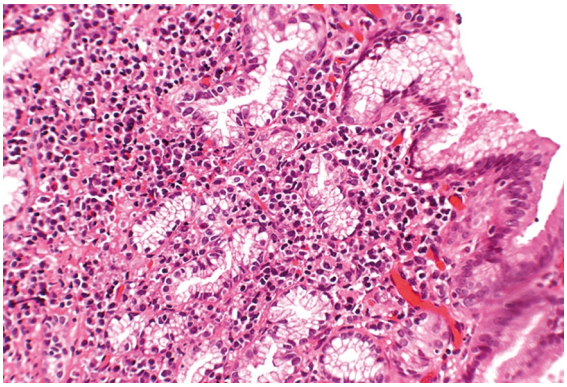

Figure 1 Gastric heterotopic tissue with evidence of inflammation/gastritis.

extraintestinal heterotopic gastric tissue with inflammation simulating appendicitis. The 2004 literature review by von Rahden *et al*[1] noted that heterotopic gastric mucosa has been found in the upper esophagus where it can lead to inflammation and esophageal webbing. A heterotopic gastric mucosa has also been found in Meckel's diverticula. According to Cserni[3] the reflux-type gastritis or gastropathy in Meckel's diverticula may account for some symptoms that have prompted removal of appendixes without inflammation.

The authors have searched the literature for similar cases simulating appendicitis using search terms including (ectopic or heterotopic) and (gastric or stomach) and (abdominal pain). Case reports were found that reported patients with abdominal pain due to gastric mucosa in the gall bladder or rectum, but these were not characterized as simulating appendicitis.

Stelle *et al*[2] describes the two prominent theories regarding the origin of gastric heterotopic tissue. One suggests that this is congenitally displaced tissue and it therefore represents choristomas. Another leading theory suggests that the tissue originates from irregular differentiation of multipotential cells rather than displaced embryonic cells.

Regardless of the origin of the tissue, the tissue is known to consist of gastric parietal cells capable of secreting a physiologically effective amount of acid leading to inflammation and occasionally to ulceration. Cserni[3] notes that in most cases, the pathology does not yield evidence of *H pylori* organisms. There was no sign of *H pylori* in our specimen.

In conclusion we present what we believe to be the first case of extraintestinal gastric heterotopic tissue simulating appendicitis. We suggest that extraintestinal gastric heterotopia be added to the list of differential diagnoses in patients with acute abdominal pain.

COMMENTS

Background

There are no cases reported in the literature wherein heterotopic gastric tissue is described as simulating acute appendicitis.

Innovations and breakthroughs

This is new information that describes a unique presentation of heterotopic gastric tissue.

Applications

Although rare, heterotopic gastric tissue could explain pain simulating appendicitis.

Terminology

Heterotopic means located in an atypical position.

Peer review

This is a case report of extraintestinal heterotopic gastric tissue simulating appendicitis. It's very interesting and worth reporting.

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CASE REPORT

Steroid responsive eosinophilic gastric outlet obstruction in a child

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Abstract

Gastric outlet obstruction is a rare complication of eosinophilic gastroenteritis, most commonly treated surgically. We report a case of eosinophilic gastric outlet obstruction in a child that responded to conservative medical management. A brief review of this clinical entity is also provided.

Key words: Eosinophilic gastroenteritis; Pylorus; Gastric outlet obstruction; Steroids

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Kellermayer R, Tatevian N, Klish W, Shulman RJ. Steroid responsive eosinophilic gastric outlet obstruction in a child. *World J Gastroenterol* 2008; 14(14): 2270-2271 Available from: URL: http://www.wjgnet.com/1007-9327/14/2270.asp DOI: http://dx.doi.org/10.3748/wjg.14.2270

INTRODUCTION

Eosinophilic gastroenteritis or gastroenteropathy (EG) is defined by variable gastrointestinal symptoms and eosinophilic infiltration of one or more areas of the gastrointestinal tract, without evidence of parasitic or extra-intestinal disease[1]. It can be idiopathic, related to food allergies, infections, and rarely infantile inflammatory bowel disease[2]. A very rare complication of this entity is distal gastritis leading to gastric outlet obstruction that has been reported to occur in infancy accompanying, mimicking or generating hypertrophic pyloric stenosis[3,4] and in adults[5,6], but has not been described in children as an isolated manifestation of EG. Most commonly patients with eosinophilic gastric outlet obstruction have been treated surgically[3,5,7] except for a few infantile cases where protein hydrolysate formula[2] or steroid therapy[4,8] has been used. Resolution of symptoms with steroid treatment has recently been demonstrated in an adult case also[6]. We report a 2 and half-year-old Caucasian girl with eosinophilic gastric outlet obstruction treated successfully with steroids.

CASE REPORT

The previously healthy girl presented after a 3-mo history of worsening postprandial emesis leading to an inability to tolerate feedings. She had no history of atopy or diet change. She had 0.08 eosinophils (740×10^6 /L) on peripheral blood count, but was not anemic nor hypoalbuminemic. IgE level was normal. Upper gastrointestinal imaging showed marked pyloric narrowing (Figure 1). Endoscopy revealed antral edema and severe pyloric stenosis through which a Pentax 2470 endoscope (8.0 mm diameter) could not be passed. Biopsies from this area were consistent with eosinophilic gastritis (Figure 2A). Methylprednisolone (2 mg/kg per day) was started and she began tolerating liquids within two days. Endoscopy five days later revealed decreased pyloric swelling and the endoscope could be passed through the pylorus. No pyloric ulceration was seen and the duodenum appeared normal. Antral and pyloric mucosal biopsies showed resolution of the eosinophilic cellular infiltrate (Figure 2B). She was advanced to a low roughage diet within a few days, switched to oral prednisolone therapy (0.5 mg/kg per day) and discharged home. The steroids were weaned and discontinued after 8 weeks of treatment. She remained symptom free six months following the cessation of steroids.

DISCUSSION

Reported clinical manifestations of eosinophilic gastroenteritis include obstruction at various levels of the

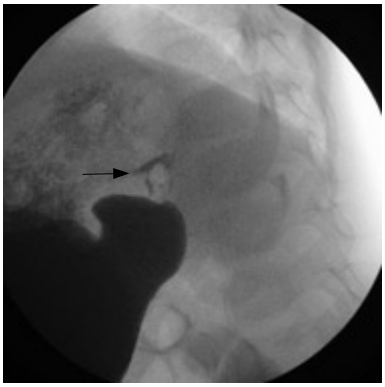

Figure 1 Upper gastrointestinal image of the patient. Note the minimal advancement of the contrast material through the pylorus (arrow).

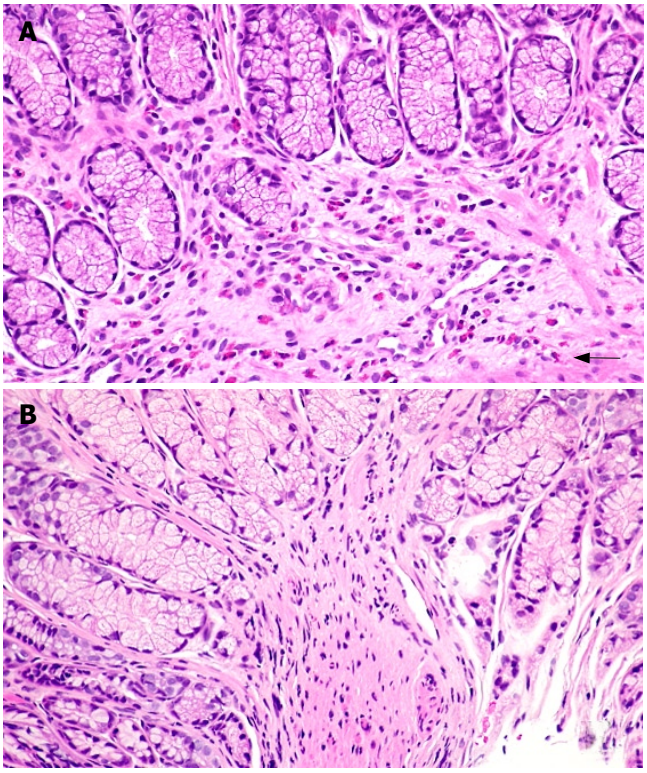

Figure 2 Histologic images before and 5 d after steroid therapy. **A**: Peripyloric antral sections showed prominent eosinophilic infiltration of the lamina propria (up to 30 eosinophils per single high power field), with occasional degranulation (arrow) of eosinophilic content and infiltration of the muscularis mucosae; **B**: Biopsies 5 d after intravenous steroid therapy demonstrated only a few eosinophils with a peak count of 2 eosinophils per high power field (HE, x 40).

gastrointestinal tract, growth failure, weight loss, anemia, melena, diarrhea, protein loosing enteropathy, abdominal pain, pseudo-Crohn's disease, esophagitis, and eosinophilic ascites[9]. On rare instances EG can even be complicated by gastrointestinal perforation[10]. While eosinophilic inflammation leading to pyloric stenosis and gastric outlet obstruction has been reported in adults and infants, it has not been described in children (pediatric patients more than 2 years of age) as a localized manifestation of EG to our knowledge. We could only identify one earlier case of antral web related gastric outlet obstruction that was complicated by eosinophilic inflammation in a 3-years-old child[11]. Our patient responded briskly to steroid therapy and has remained asymptomatic for more than six months off therapy. Similar clinical response has been recorded earlier in infants with the same condition[4,8] and very recently in an adult[6]. However, in several instances resolution of the eosinophilic inflammation can be protracted and the course of the disease may wax and wane[4,10]. Nevertheless, we conclude that steroid therapy should be considered in cases of eosinophilic gastric outlet obstruction prior to surgical interventions in all age groups.

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CASE REPORT

Ischemic colitis due to obstruction of mesenteric and splenic veins: A case report

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Abstract

Ischemic injury to the bowel is a well known disease entity that has a wide spectrum of pathological and clinical findings. A sudden drop in the colonic blood supply is essential to its development. We encountered a 41-year-old male patient, who presented with abdominal pain and bloody diarrhea. A colonoscopy showed markedly edematous mucosa with tortuous dilatation of the veins and a deep ulceration at the rectosigmoid junction. On an abdominal computed tomography (CT) scan and CT angiography, the mesenteric and splenic veins were absent with numerous venous collaterals for drainage. The patient gradually responded to oral aminosalicylate therapy, and was in remission after nine months. In most cases, non-occlusive ischemic injury is caused by idiopathic form and occlusive ischemia is caused by abnormalities of arteries and acute venous thrombosis. However, chronic venous insufficiency due to obstruction of macrovascular mesenteric vein rarely causes ischemia of the bowel. This report describes the first case of ischemic colitis caused by obstruction of the mesenteric and splenic veins.

Key words: Ischemic colitis; Mesenteric vein; Splenic vein

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Hwang SS, Chung WC, Lee KM, Kim HJ, Paik CN, Yang JM. Ischemic colitis due to obstruction of mesenteric and splenic veins: A case report. *World J Gastroenterol* 2008; 14(14): 2272-2276 Available from: URL: http://www.wjgnet.com/1007-9327/14/2272.asp DOI: http://dx.doi.org/10.3748/wjg.14.2272

INTRODUCTION

Ischemic colitis is the most common ischemic injury of the gastrointestinal tract. Although it can occur at any age, approximately 90% of the patients were over 60 years of age[1]. It is usually self-limited and is often called transient ischemic colitis.

We encountered a 41-year-old male patient, who presented with abdominal pain and bloody diarrhea, and had been diagnosed as having ulcerative colitis. The colonoscopy showed ulcerative colitis, but the rectum was spared from inflammation. Corticosteroids did not relieve the disease, but aggravated the symptoms. On an abdominal computed tomography (CT) scan and CT angiography, the mesenteric and splenic veins were absent with numerous venous collaterals for drainage. The final diagnosis was ischemic colitis with obstruction of the mesenteric and splenic veins. In this report, we describe this unusual case of ischemic colitis caused by chronic venous insufficiency.

CASE REPORT

A 41-year-old man, a Bangladeshi migrant worker in Korea, was admitted to our hospital with a three-year history of lower abdominal pain and bloody diarrhea. He was diagnosed with ulcerative colitis, treated with oral corticosteroids in an outside clinic, and referred to our facility for further evaluation. The physical examination was remarkable for lower abdominal tenderness without rebound. A routine blood analysis revealed a slightly decreased level of hemoglobin (109 g/L), hematocrit (33.8%), white cell count (14.12×10^9/L) and platelets (106×10^9/L). Urea and electrolytes, glucose, amylase and liver function were normal but the patient had a slightly raised level of C-reactive protein (6.7 mg/L). The patient was presumed to have ulcerative colitis with moderate to severe activity and was treated with corticosteroids. After 48 hours, he underwent a flexible sigmoidoscopy, which showed a 3-cm sized deep ulceration at the rectosigmoid junction, and friable mucosa in a diffuse circumferential distribution. Severe colitis with superimposed infection was

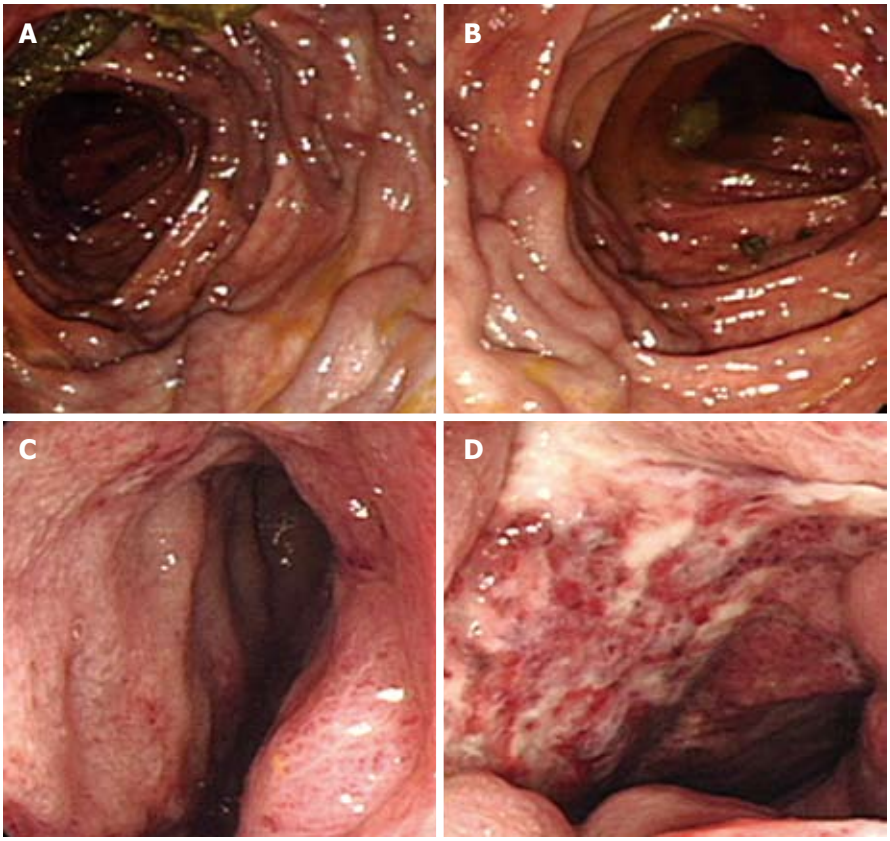

Figure 1 Colonoscopic findings. **A**, **B**: Tortuous and dilated submucosal veins in the colonic mucosa; **C**: Diffuse and edematous mucosa with fine granularity resembling ulcerative colitis; **D**: A deep ulceration at rectosigmoid junction.

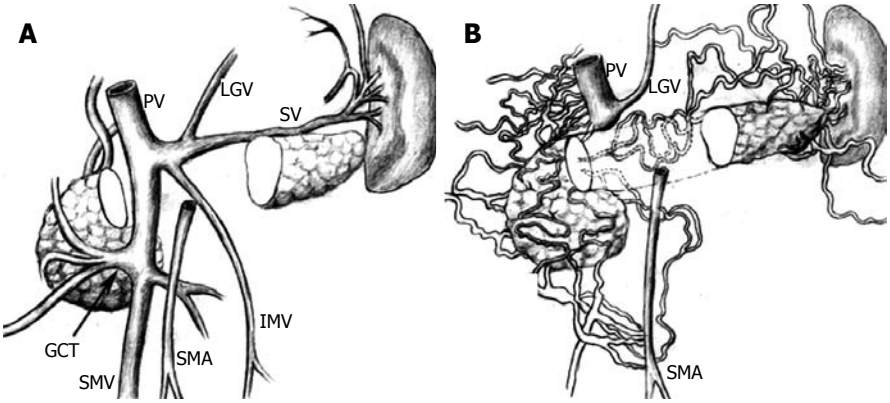

Figure 2 Dynamic abdominal CT. **A**: The normal anatomy of the main portal vein and its tributaries including the portal vein (PV), left gastric vein (LGV), gastrocolic trunk (GCT), splenic vein (SV), SMA, SMV, and IMV are schematically illustrated; **B**: The normal mesenteric vein (SMV and IMV) and splenic vein are absent. Numerous collateral venous channels developed to drain venous blood from gastrointestinal tract to portal vein.

suspected because of mild fever, and antibiotic treatment was added. Blood and stool cultures were negative. There was a minimal improvement in the clinical course, but his symptoms were not totally alleviated. Two weeks after admission, a colonoscopy was performed to evaluate the extent and other causes of the disease. It demonstrated colitis with markedly edematous mucosa and tortuous and dilated veins throughout the colon, but the rectal mucosa was spared from inflammation. The fine granularity of the mucosa was associated with friability, and contact bleeding with mucus was observed (Figure 1A-D). The biopsy specimens obtained from the edematous colonic mucosa and the ulcer of the rectosigmoid junction showed nonspecific chronic inflammation.

A dynamic abdominal CT was performed. On arterial phase CT, diffuse edematous thickening of the colon with dilated and tortuous peripheral branches of colic arteries was seen. The rectosigmoid colon appeared markedly thickened. Major abdominal arteries including the celiac axis, splenic artery, superior mesenteric artery (SMA) and inferior mesenteric artery (IMA) were all normal in size and shape. However, on venous phase CT, the splenic vein, superior mesenteric vein (SMV) and inferior mesenteric vein (IMV) were absent. Dilated and tortuous peripheral colic veins drained into the portal vein via tortuously dilated intraperitoneal collaterals (Figures 2A and B, and 3A-C). Venous blood of gastrointestinal tract and spleen drained into the portal vein via various routes of the collateral venous pathway that are similar to the intra-abdominal venous collaterals in patients with portal hypertension. The main collateral venous routes in our patient were pericolic and mesenteric collaterals, splenic hilar and perigastric collateral-pancreaticoduodenal collaterals-the portal vein. Some venous drainage of the lower portion of the left

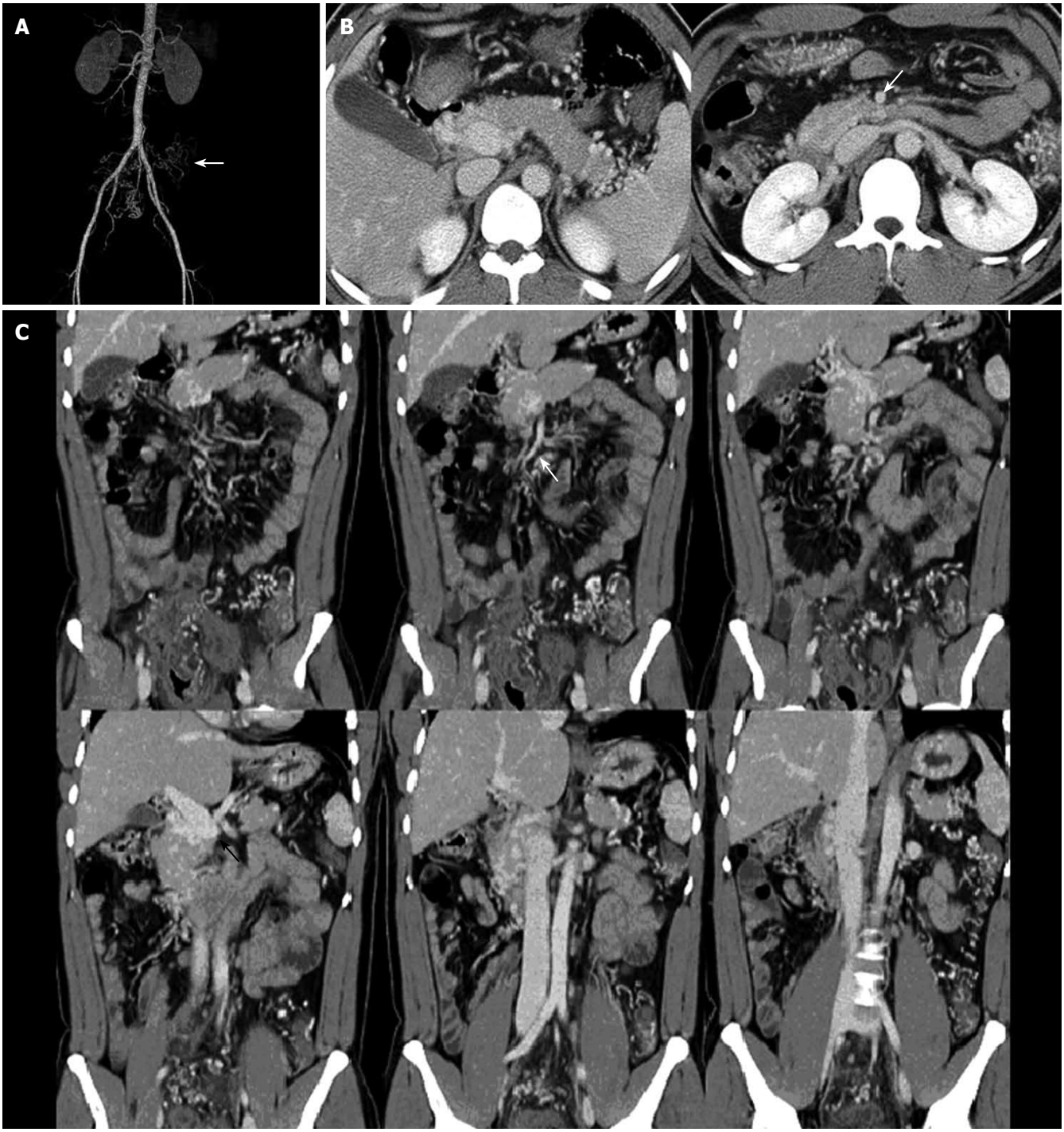

Figure 3 Abdominal CT arteriography. **A**: Normal shape and course of SMA, IMA, and splenic artery, tortuous and dilated distal branches of SMA and IMA around rectosigmoid colon; **B**: An axial and **C**: Coronal reformatted abdominal CT reveal the absent splenic vein, SMV, and IMV with numerous intra- and peripancreatic collaterals. Distal end portal vein is abruptly ended (black arrow) and SMA is normal (white arrow). Note prominent dilated pericolic arteries and venous collaterals, and markedly thickened sigmoid colon.

colon drained into the left renal vein via the left gonadal vein. In addition, the venous collaterals of the rectosigmoid colon drained into the bilateral internal iliac veins. The patient refused angiographic evaluation for the anomalous abdominal venous system.

The final diagnosis was ischemic colitis with obstruction of the mesenteric and splenic veins. Corticosteroids did not relieve the disease, but aggravated the symptoms. The patient discontinued steroid therapy and gradually responded to oral aminosalicylates. After nine months, he was in remission and re-evaluated by sigmoidoscopy. It showed some improvement of the colonic inflammation and a complete resolution of the ulcer in the rectosigmoid area.

DISCUSSION

Ischemic colitis is a well-recognized clinical phenomenon,

although its precise etiology remains unclear. It may manifest a spectrum of severity from mild, transient mucosal erosion to fibrous scarring with stricture formation and even transmural infarction. Some cases are caused by acute macrovascular mesenteric occlusion due to surgical trauma[2], thromboembolism[3-5], or atherosclerosis[6]. However, chronic venous insufficiency is rarely associated with ischemic colitis. Ischemic colitis typically develops spontaneously without signs of major vascular occlusion, and viable intestine is present elsewhere in the tract. Isolated case reports have described development of ischemic colitis in conjunction with mild allergy, hypertension, rectal prolapse, acute pancreatitis, sickle cell crisis, colon cancer, systemic lupus erythematosus, amyloidosis, anticardiolipin antibody syndrome, Buerger's disease, and Kawasaki syndrome[7-9]. Other case reports described the association between development of ischemic colitis and use of some agents (progesterone, ergotamine derivatives, nonsteroidal anti-inflammatory drugs, and danazol)[10], intravenous vasopressin therapy[11], renal transplantation[12], chronic intermittent peritoneal dialysis[13], cocaine abuse, snake bite and marathon running[7].

Clinical presentation is usually acute, with cramping abdominal pain of abrupt onset, abdominal distention, and bloody diarrhea. There may be local signs of peritoneal irritation over the affected segment, and if mucosal ulceration is present, bacterial invasion may also occur. However, manifestations vary widely, from severe pain with transmural infarction and early perforation to mild abdominal pain and only slight tenderness[14].

It is extremely difficult to differentiate ischemic from ulcerative colitis. Moreover, ischemic and idiopathic ulcerative colitis may coexist[15]. An endoscopic finding of ulcerative colitis is characterized by a uniform inflammatory reaction in the colonic mucosa, without intervening areas of normal mucosa. The majority of cases arise in the rectum, and some authorities believe that the rectum is always involved in an untreated patient[16]. With inflammation, the mucosa becomes erythematous and granular, and the vascular pattern becomes obscured by edema. In this patient, a similar pattern of mucosal lesions was observed as mentioned above. However, while making the diagnosis as ulcerative colitis, we found that (1) the rectal mucosa was free from inflammatory reaction; (2) although the patient did not have a fulminant clinical course, a deep ulceration was observed; and (3) the disease was resistant to corticosteroid therapy and instead aggravated his clinical course. Color Doppler scans were used to differentiate the bowel-wall thickening in ischemic colitis from that seen in inflammatory bowel disease[7].

Generally, major arterial or venous branches are easily detected on arterial or venous phase CT. The SMV is located anteriorly and to the right of the SMA and posteriorly medial to the head of the pancreas. The SMV tributaries are the ileocolonic, pancreatoduodenal, and gastroepiploic veins. The IMV originates anterior to the sacrum as the superior rectal (hemorrhoidal) vein and receives branches from the sigmoid and descending colon as it ascends to the left of midline, adjacent to the inferior mesenteric artery and left gonadal vein. In the upper abdomen, the IMV passes from posterior to the distal duodenum, anterior to the left renal vein, and then anterior to the SMA before anastomosing with the portal venous system. The splenic vein is easily detected beneath the pancreas and drains to the portal vein[17]. In this patient, the proximal SMV, IMV and most of splenic vein were absent despite the presence of a normal SMA and IMA and splenic artery. The tortuous and dilated distal branches of SMA and IMA around the entire colon were seen on arterial phase CT and no remarkable SMV and IMV were noted on venous phase CT except for the prominent collateral veins. Instead of a normal splenic vein beneath the pancreas, tortuous splenic hilar venous collaterals developed and drained into the portal vein via the peripancreatic venous collaterals. Although we have no direct angiographic evidence, the anomaly described in this report appears unique. We presume the congenital absence of the SMV, IMV, and splenic vein results from excessive involution of proximal vitelline veins.

In this case, the arterial blood supply and venous drainage might have been balanced for a long time because of numerous abdominal venous collaterals. In addition, a possible breakage of this balance may cause venous stasis and ischemia of the gastrointestinal tract. Impaired colonic venous drainage may be a possible cause or vulnerable to the development of ischemic colitis. The relationship between the absence of mesenteric veins with possible venous stasis and ischemic colitis is not clearly established. Although the patient is currently doing well after medical and conservative treatment, a long-term follow-up is needed as there is little information in the literature regarding the outcome of the absence of the proximal mesenteric veins and its influence upon venous drainage.

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CASE REPORT

Successful endoscopic treatment of biliary stricture following mesenteric tear caused by blunt abdominal trauma

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Abstract

Biliary duct injuries are frequently iatrogenic, being associated with surgery for gallbladder stones. However, blunt abdominal trauma such as a motor vehicle crash is a rare cause of extrahepatic biliary stricture. A few reports have been published on biliary strictures treated with endoscopic therapy. In the present study, we describe a suprapancreatic biliary stricture associated with mesenteric tear following road traffic accident. We performed endoscopic stent placement, which was successful in relieving the biliary stricture.

Key words: Biliary stricture; Blunt abdominal trauma; Mesenteric tear; Endoscopic stent treatment

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Kang DO, Kim TH, You SS, Min HJ, Kim HJ, Jung WT, Lee OJ. Successful endoscopic treatment of biliary stricture following mesenteric tear caused by blunt abdominal trauma. *World J Gastroenterol* 2008; 14(14): 2277-2279 Available from: URL: http://www.wjgnet.com/1007-9327/14/2277.asp DOI: http://dx.doi.org/10.3748/wjg.14.2277

INTRODUCTION

Benign bile duct strictures are uncommon and usually follow surgery for gallbladder stones, both open surgery and laparoscopic surgery[1]. However, development of biliary stricture after blunt abdominal trauma is an extremely rare condition[2-4]. No definitive treatment has been proposed for biliary strictures caused by blunt abdominal trauma. Recently, nonsurgical intervention using endoscopic approach has been used, with promising results[4,5]. We present a patient with a suprapancreatic biliary stricture that developed following a mesenteric tear caused by blunt abdominal trauma due to a traffic accident. The patient was treated successfully with endoscopic plastic stent placement.

CASE REPORT

A 36-year-old man had blunt abdominal trauma due to a traffic accident. The patient was the driver and suffered full impact with the steering wheel. The patient was hospitalized for the treatment of hemoperitoneum caused by a mesenteric tear. Surgery was performed and the mesenteric tear was repaired. Two weeks after the accident, the patient developed jaundice and was transferred to our hospital for further evaluation and management.

At admission, the patient had overt jaundice with itching over the entire body. The vital signs were stable. Physical examination showed no abdominal pain, or tenderness to palpation. The bowel sounds were normal. The initial liver function tests were as follows: aspartate aminotransferase 60 U/L (normal range, 0-37 U/L), alanine aminotransferase 92 U/L (normal range, 0-41 U/L), alkaline phosphatase 317 U/L (normal range, 35-130 U/L), gamma-glutamyl transferase 107 U/L (normal range, 8-61 U/L), total bilirubin 16.9 mg/dL (normal range, 0-1.2 mg/dL), and direct bilirubin 11.3 mg/dL (normal range, 0-0.3 mg/dL). The amylase and lipase levels were within the normal range. Computer tomography (CT) scan of the abdomen revealed dilatation of both intrahepatic (IHD) and extrahepatic biliary ducts (EHD) with abrupt cut-off of the suprapancreatic portion of the common bile duct (CBD) (Figure 1). There was no evidence of pancreatitis.

An endoscopic retrograde cholangiopancreatography (ERCP) was performed to determine the cause of the jaundice. The cholangiogram revealed a 1 cm long stenosis of the distal CBD just above the pancreas, with ductal dilatation above the narrowed portion. Endoscopic biliary sphincterotomy using a pull-type papillotome and brushing and biopsy of the narrowed part were performed (Figure 2). At the end of the procedure, an endoscopic nasobiliary drain (ENBD) was placed. After the procedure, the serum

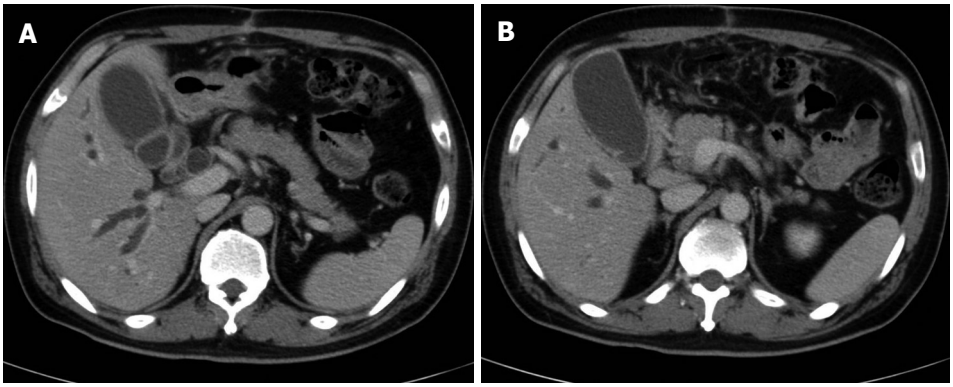

Figure 1 Abdominal CT scan findings. **A**: Dilated intra- and extrahepatic bile ducts; **B**: Abrupt cut-off of the distal common bile duct.

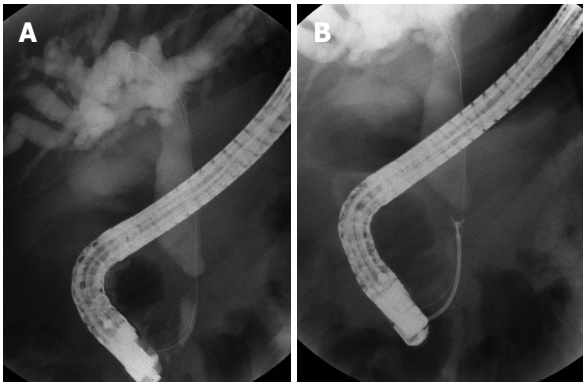

Figure 2 ERCP findings. **A**: Stenosis of the distal CBD, approximately 1 cm in length, located just above the pancreas, with ductal dilatation above the narrowed portion; **B**: Biopsy of the stricture was obtained.

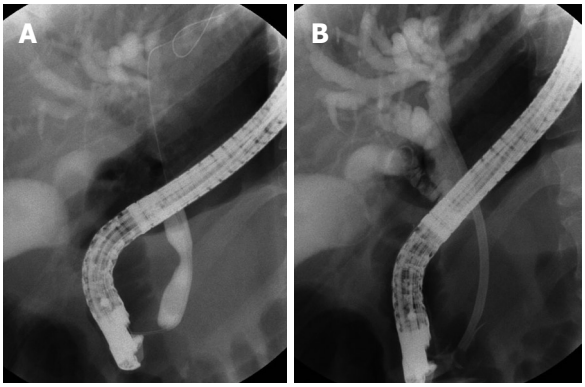

Figure 3 ERCP findings. **A**: The CBD was dilated with TTS balloons, but the dilatation was not effective; **B**: An Amsterdam-type plastic stent (12Fr, 7 cm) was placed across the stricture.

bilirubin levels decreased gradually. The cytology and biopsy results were negative for malignancy.

A repeat ERCP was performed 5 d after the first procedure and the CBD was dilated with TTS balloons. However, the stricture could not be dilated effectively. Therefore, a 12 Fr. plastic stent, 7 cm in length, was inserted and placed at the site of the lesion (Figure 3). Three months later, the patient was readmitted for a follow-up ERCP. The liver function tests were within the normal range and the patient did not have jaundice. The cholangiogram showed improvement of the CBD stricture. The plastic stent was removed after observing normal passage of radiocontrast material (Figure 4). During a follow-up period of 26 mo, there has been no recurrence of the biliary stricture as judged by laboratory tests and radiological imaging studies.

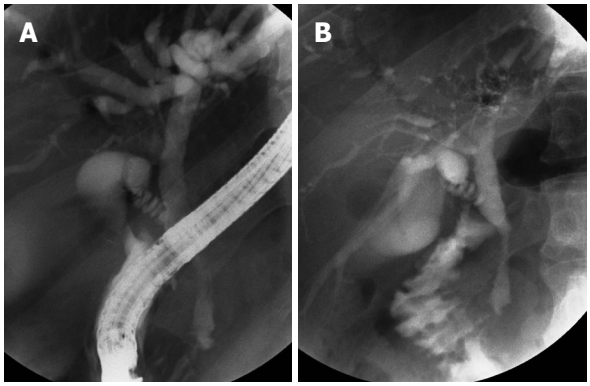

Figure 4 Follow-up ERCP findings. **A**: Improvement of the CBD stricture; **B**: There is normal passage of radiocontrast material into the bile duct through the papilla.

DISCUSSION

The bile ducts are located deep within the abdomen and are protected by the ribs, liver and mesentery. Therefore, non iatrogenic bile duct injuries secondary to blunt abdominal trauma are extremely rare. It is most commonly seen following motor vehicle accidents when an unrestrained driver has an impact with the steering wheel[2-4].

The proposed mechanisms of extrahepatic biliary strictures after blunt trauma differ based on their location. The possible mechanisms underlying the development of a suprapancreatic biliary stricture are: (1) local inflammation followed by fibrosis and stricture formation secondary to ductal tear; (2) disruption of the blood supply to the bile duct, and (3) compression of the biliary duct by an intramural or extrabiliary hematoma[3-5]. The possible mechanisms for the development of an intrapancreatic biliary stricture include the following: posttraumatic pancreatitis with swelling of the pancreatic head, and compression of the intrapancreatic portion of the CBD; this usually resolves as the swelling of the pancreas subsides[6]. We believe that the suprapancreatic biliary stricture in our patient was caused by disruption of the blood supply and extrinsic compression by a hematoma. The hepatobiliary area was not entered during the surgery, thus reducing the

possibility of an iatrogenic trauma.

Most patients with a biliary stricture secondary to blunt abdominal trauma develop jaundice as the initial symptom. The onset of symptoms is insidious[3,5]. The condition may be mistaken for cholangiocarcinoma, if history of abdominal trauma is not available[7], and may result in unnecessary surgery.

The correct diagnosis may be difficult to determine based on imaging studies such as abdominal CT and ERCP, in the absence of a complete history[5,7,8]. Therefore, a high index of suspicion is required to ensure a proper diagnosis. To prevent irreversible fibrosis, an early diagnosis of biliary stricture is essential in order to allow timely treatment with percutaneous or endoscopic intervention[3,5,9]. Several workers have suggested that endoscopic stent placement is an effective treatment for suprapancreatic biliary strictures caused by blunt abdominal trauma[5,10,11]. Park *et al*[5] reported eight patients with suprapancreatic biliary stricture due to blunt abdominal trauma. The median length of the stricture was 1 cm. Balloon dilatation was performed in only 1 patient. The median duration of plastic stent placement was 2.9 mo. The patients were all successfully treated with the endoscopic stent placement and there was no recurrence during the follow-up period (median 33 mo). Our case was similar in terms of clinical features and outcome compared to previously reported cases. Endoscopic stenting for biliary stricture after blunt abdominal trauma is associated with low morbidity and excellent long-term outcome[5,10,11]. Therefore, endoscopic stenting is the treatment of choice for biliary strictures that develop after blunt abdominal trauma. Surgery should only be undertaken if endoscopic intervention fails[12,13].

In summary, we report a patient with a suprapancreatic biliary stricture secondary to blunt abdominal trauma, which was treated successfully with endoscopic stent placement. The excellent results obtained in our patient suggest that endoscopic stent placement should be considered as the primary treatment for patients who develop an extrahepatic biliary stricture after blunt abdominal trauma. Endoscopic treatment is safe and has a favorable long-term outcome.

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CASE REPORT

Appendiceal mucocele: Case reports and review of current literature

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Abstract

The mucocele of the appendix is an uncommon disorder which is often asymptomatic but sometimes causes acute appendicitis-like symptoms. Sometimes, patients with mucocele can present with confusing symptoms. Preoperative suspicion and diagnosis of appendiceal mucocele are important. Ultrasonography and computed tomography are useful tools for the diagnosis of appendiceal mucocele. It may be also recognised by colonoscopy as a smooth submucosal lesion of the cecum. Optimal management of the mucocele could be achieved through accurate preoperative diagnosis. Preoperative diagnosis is a major component for minimizing intra-operative and post-operative complications. We herein report five cases and discuss the diagnostic methods and surgical treatment.

Key words: Appendix; Mucocele; Diagnosis; Surgical treatment

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INTRODUCTION

Appendiceal mucocele (AM) is a rare entity that can present with a variety of clinical symptoms or occur as an incidental surgical finding. The incidence is 0.2%-0.4% of all appendectomied specimens[1-3]. AM is a progressive dilatation of the appendix from the intraluminal accumulation of the mucoid substance[3,4]. It may be a benign or malignant process. There are four histological types, which lead to individualized surgical treatment and course in each case[3].

Preoperative diagnosis that distinguishes AM from acute appendicitis (AA) is essential for the best choice of surgical approach (open *vs* laparoscopic) to prevent peritoneal dissemination and perform the appropriate surgery[3,5]. Herein, we report 5 cases, discuss the diagnostic procedures and management algorithm of these patients, and review briefly the relevant literature. We also aimed to define the incidence of the AM in a tertiary referral centre in Northern Black Sea region of Turkey.

CASE REPORTS

Case 1

An 82-year-old man was admitted to the hospital because of pain in the right lower quadrant of the abdomen for 3 d. Standard laboratory tests, serum levels of CA 19-9 and carcino-embryonic antigen (CEA) were within normal ranges. Ultrasonography (USG) and computerized tomography (CT) demonstrated a well demarcated, elliptical 7 cm × 5 cm cystic mass with parietal calcifications in the right lower quadrant of the abdomen. There was an indentation in the cecum by colonoscopy. Surgical exploration revealed the mass to be an AM. Simple appendectomy was performed. Pathological examination revealed a mucinous cystadenoma with dimensions of 8 cm × 6 cm × 5.5 cm. AM restricted to the appendix and cecum was free of the disease. The patient's postoperative course was unremarkable, and he was discharged home on the 4th postoperative day.

Case 2

A 65-year-old woman was referred to the emergency de-

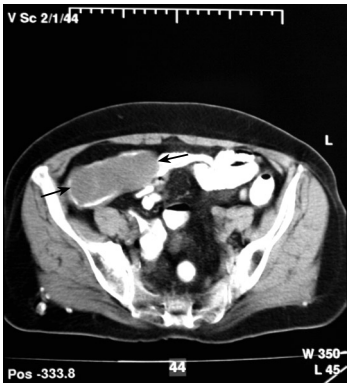

Figure 1 CT imaging of a soft tissue mass indicated by black arrows in the region of the cecum.

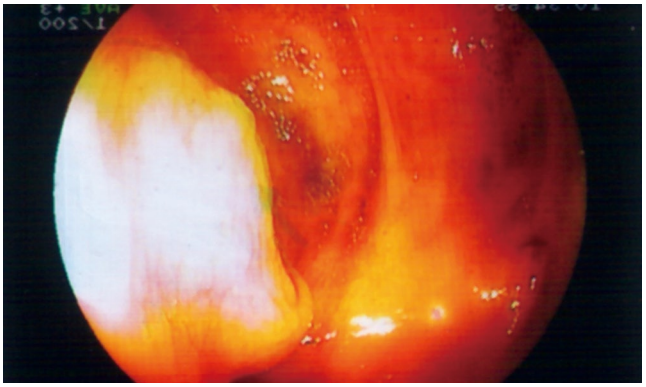

Figure 2 Colonoscopic view of the sub-mucosal mass.

partment with a diagnosis of AA. She complained of an abdominal pain which started 3 d before referral to our hospital. She had a history of coronary by-pass surgery 3 years ago. Physical examination showed diffuse peritoneal irritation. Plain radiography showed gas-fluid levels in the right lower quadrant. She also had mild leukocytosis. USG and CT scans of the abdomen showed presence of free fluid in the intra-abdominal spaces.

An emergency operation revealed ileal and cecal mesenteric ischemia. Partial ileum and cecum resection was performed. Histopathological diagnosis was ischemia of the intestine and simple mucocele of the appendix with a diameter of 5 mm, incidentally. Postoperative recovery was uneventful and she was discharged home on the 7th postoperative day.

Case 3

A 66-year-old woman was referred to the endoscopy unit for evaluation of a mass in the right lower quadrant of the abdomen with the suspicion of malignant tumor. She had visited another health care unit where the mass was revealed by ultrasound examination. She had been suffering from decreased appetite, nausea, and weakness lasting for a week. She had a history of upper GI hemorrhage managed medically 16 years ago and her surgical history was significant for cholecystectomy 14 years ago. Routine laboratory tests, including complete blood count and serum chemistry were unremarkable. Serum levels of CA19-9 and CEA were also within the normal ranges. USG of the abdomen showed a heterogenic mass (95 mm × 40 mm × 32 mm in dimensions) involving ileocecal part of the intestines. CT imaging revealed a soft tissue mass measuring 8 cm × 4 cm with peripheral enhancement in the region of the cecum (Figure 1). The patient underwent colonoscopy, which revealed a sub-mucosal protrusion to the lumen of the cecum, in the region of the appendiceal orifice. Orifice of the appendix was uncertain (Figure 2). Remaining of the colon was unremarkable. The patient was treated surgically. Surgical exploration revealed a mass in the appendix whitish-grey in color (Figure 3). Resection of cecum was followed by an ileocolic anastomosis. Frozen section examination revealed benign mucoid lesion. Formal pathologic report was hyperplastic type mucocele with evidence of secondary changes and chronic inflammatory mucosa of the intestine. The mucocele was 9.5 cm × 4 cm × 2 cm in dimensions with a wall thickness of 2 mm. There was no evidence of malignancy. Postoperative course was unremarkable and she was discharged home on the 5th postoperative day.

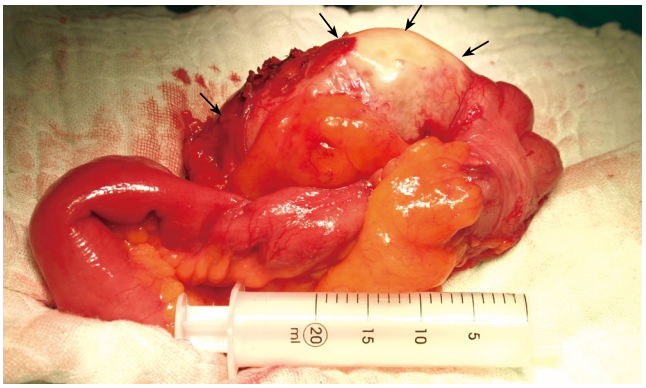

Figure 3 Intra-operative view of the AM. Arrows indicate the mucine filled appendix.

Case 4

A 58-year-old man was referred to the general surgery department for surgical treatment of toxic multi-nodular goitre. He had multiple previous admittances to internal medicine department and emergency department because of pain in the right lower quadrant of the abdomen and anemia. Physical examination was negative for abdominal mass or perianal or rectal lesions. The patient underwent endoscopy to investigate the etiology of anemia. Colonoscopy revealed a submucosal cecal mass. Abdominal CT revealed a polipoid a soft tissue mass measuring 4 cm × 3 cm with peripheral enhancement in the region of the cecum. The patient underwent total thyroidectomy and appendectomy. Histopathological diagnosis was benign cystadenoma without cecal involvement. Postoperative course was unremarkable and he was discharged home on the 6th postoperative day.

Case 5

A 72-year-old man underwent an open inguinal herniorrhaphy due to recurrent right inguinal hernia. He had complained of bulging and right groin pain which were exacerbated with activity. An appendiceal mass was defined during laparoscopic herniorrhaphy. Open access to the

abdominal cavity was chosen. Simple appendectomy was performed with a clinical suspicion of appendiceal mucocele. Frozen section examination revealed appendiceal mucocele. Pathological examination reported a mucinous cystadenoma measuring 12.5 cm and 5.5 cm without cecal involvement. Postoperative course was unremarkable and he was discharged home on the 7th postoperative day in good conditions.

DISCUSSION

Mucocele of the appendix is an uncommon tumor, with an incidence of 0.29%-0.4% of all appendectomied specimens[1-3,6]. There has been no exact reported incidence of AM in Turkey. Histopathological examinations revealed 5 patients with AM among 240 patients who underwent appendectomies from January 2001 to October 2007 at our institution. The incidence of AM is revealed as 2.01%, which is higher than that reported in literature[7,8]. This may be related to the fact that our centre is a tertiary referral centre.

Although a small portion of AM is asymptomatic, clinical manifestations include right lower abdominal pain, palpable abdominal mass, or gastrointestinal bleeding in majority of the AM[1,3,7,9-12]. Among the five cases reported above, three had abdominal pain secondary to mucocele while one had symptoms related to groin hernia and the last patient had abdominal pain due to intestinal infarction. AM was an incidental finding for those two patients.

USG, CT and colonoscopic examinations can facilitate preoperative diagnosis of AM[1,13-15]. Ultrasound is the first-line diagnostic modality for patients with acute abdominal pain or mass. Different sonographic findings of AM and AA have been described[3,16,17]. Appendix diameter 15 mm or more in USG examination has been determined as the threshold for AM diagnosis with a sensitivity of 83% and a specificity of 92%[3]. Outer diameter threshold for AA diagnosis has been established as 6 mm[18]. USG examination revealed a cystic mass in the right lower quadrant in two of our patients. These findings revealed suspicion of AM.

CT is also an effective diagnostic tool for AM. CT can determine the relation between lesion and the neighbouring organs, and help confirm the diagnosis[15,16,19,20]. CT reveals a cystic mass with enhancing wall nodularly in the expected area of the appendix, especially in older patients, in whom AM should be considered[9,15,16,21]. AM could be visualized by CT in three of our patients. CT examination was normal in respect of AM for the third patient. AM was an incidental finding for this lady. Radiological investigation was not performed for the patient who had herniorrhaphy for recurrent groin hernia.

USG and CT findings are non-specific and the differential diagnosis should be established with benign appendiceal neoplasms and other pathologies such as carcinoid, lymphoma, mesenteric cysts, ovarian masses, and malign neoplasms of the appendix[7,22]. We did not perform fine needle aspiration as dictated in the literature in order to avoid dissemination of the mucus leading to pseudomyxoma peritonei[16].

Colonoscopy in patients with abdominal pain is a useful tool for determination of mucocele[2,23]. Generally, an elevation of the orifice of the appendix is seen. A yellowish mucous discharge would be visible from appendiceal orifice during colonoscopy. It is also important for the diagnosis of synchronous or metachronous colon tumor which would be as high as 29%[1,2,7,13-15,24,25]. Colonoscopic examinations on three of our patients revealed indentation in the cecum due to AM. The remaining colon was unremarkable in all of them. The 4th patient in emergency conditions was treated surgically based on the diagnosis of mesenteric ischemia and the 5th patient underwent surgery with the diagnosis of groin hernia without colonoscopic examination.

The spontaneous and surgery induced complications of AM include intestinal obstruction, intussusceptions[20,22], intestinal bleeding[11,13,25], fistula formation[15,26], and volvulus[27,28]. The worst complication is pseudomyxoma peritonei, characterized by peritoneal dissemination caused by iatrogenic or spontaneous rupture of the mucocele[5]. The tissues should be handled carefully during surgery in order to avoid rupture of the mucocele. Thus, conventional surgery is preferred rather than laparoscopic approaches for the treatment in our cases[2,5,8,22]. Laparoscopic approach has an increased risk of rupture and subsequent pseudomyxoma peritonei formation[2,5,8]. Moreno *et al*[5] suggest conversion to an open appendectomy in case of mucocele when laparoscopic appendectomy is intended. Few authors still recommend a minimally invasive approach in selected patients for this rare entity[9,27,29]. However, in these reports, laparoscopic approach has been adopted for a small number of patients. Thus, we need a large series to substantiate recommendations of laparoscopic approach.

A simple and thorough evaluation of these patients with a new algorithm has been suggested by Dhage-Ivatury and Sugarbaker[30]. Simple appendectomy is the choice of surgical treatment for patients with benign mucocele that has negative margins of resection without perforation. No long term follow-up is needed for these patients[2,8,27,30]. Appendectomy was performed for three of our patients with a mucocele limited to the appendix. For patients with perforated mucocele, with positive margins of resection, positive cytology and positive appendiceal lymph nodes, right colectomy/cytoreductive surgery (CRS)/heated intraperitoneal chemotherapy (HIIC) and early postoperative intraperitoneal chemotherapy (EPIC) should be performed. Long term follow-up is obligatory for these patients[5,24,30,31].

Perforated mucocele with positive margins of resection, positive cytology, and negative appendiceal lymph nodes necessitate cecectomy/CRS/HIIC and EPIC. Long term follow-up is also obligatory for these patients[2,30,32]. Cecectomy had been performed for one of the patients to obtain negative surgical margins, since the appendiceal wall was contiguous with the cecum and the intraoperative pathology indicated benign mucocele. Long term follow-up in this case has been carried out in the outpatient clinics. Perforated mucocele with positive cytology but negative margins of resection and negative appendiceal lymph nodes should be treated with appendectomy/CRS/HIIC and EPIC[26,30]. We did not apply these treatment modali-

ties as none of our patients had had positive lymph nodes or perforated mucocele.

CONCLUSION

Although a rare disease, surgical treatment of the AM is mandatory because of the potential for malignant transformation and prevention of pseudomyxoma peritonei due to rupture of the mucocele itself. Therefore, preoperative diagnosis or suspicion is required for carefully planned resection to excise the tumor. The incidence of AM in a tertiary referral centre in Northern Black Sea region of Turkey is revealed as 2.01%, which is higher than the incidence reported in the literature.

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CASE REPORT

Tuberculous abscess in hepatoduodenal ligament: Evaluation with contrast-enhanced computed tomography

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Abstract

Two patients with tuberculous abscess in the hepatoduodenal ligament were studied. Both patients underwent contrast-enhanced computed tomography (CT) scan. The abscess showed a low density with an irregular thick wall in the hepatoduodenal ligament on CT images, the margin was poorly defined. Contrast-enhanced CT images showed the contrast-enhanced thick wall, homogeneous and peripheral-enhanced lymph nodes. Although features of the tuberculous abscess in the hepatoduodenal ligament could be conspicuously shown with contrast-enhanced CT, further experience is needed to evaluate the potential value of CT in detecting early tuberculous abscess in relation to other entities in the hepatoduodenal ligament.

Key words: Tuberculosis; Abscess; Hepatoduodenal ligament; X-ray; Computed tomography; Lymph node

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INTRODUCTION

The incidence of tuberculosis is increasing[1-4]. Abdominal tuberculosis can affect the gastrointestinal tract, peritoneum and lymph nodes. Lymphadenopathy is the most common manifestation of abdominal tuberculosis[5,6]. To our knowledge, there are no reports on the computed tomography (CT) appearance of tuberculous abscess in the hepatoduodenal ligament in the English radiologic literature. We describe in the paper the CT findings in a series of tuberculous abscesses in the hepatoduodenal ligament.

CASE REPORTS

Case 1

A 24-year-old man had weight loss, easy fatigability, night sweats, and obscure abdominal pains for three months, but no clinically palpable abdominal mass.

Contrast-enhanced CT was performed on a 16-detector spiral CT scanner (Siemens Sensation). The image protocol consisted of dual-phase CT scan. Medrad-100 power injector was used. Contrast material was Ultravist (Schering Germany, 300 mgI/mL). The range of CT scan covered the upper and middle abdomen. CT parameters were 120 KV, 165 eff mAs, 0.5 s/360°, 16 mm × 0.75 mm, 1 mm multi-planar reconstruction (MPR) slice width. CT scan was performed with intravenous contrast material administered as a bolus. Oral contrast material (1.2% angiografin) was administrated. Images were viewed at a window width setting of 400 HU and a window level setting of 50 HU.

Contrast-enhanced CT showed a less dense mass with thick enhanced irregular walls in the hepatoduodenal ligament. The mass measured 3.9 cm × 2.8 cm with its margin poorly defined. The interface between the mass and around organs was not clear. Gas collection was not shown. Enlarged lymph nodes were detected in the portacaval space, gastrohepatic ligament, peripancreatic and upper paraaortic region. Some of them had peripheral enhancement and necrosis in the center (Figure 1A-E).

Operation on abdominal region was performed. Macroscopic pathological examination showed that the mass was tightly adherent to the hepatoduodenal ligament. Enlarged lymph nodes were found. Thirty mL of pus was aspirated from the mass. Microscopic examination revealed that the mass was inflammatory, and bacterial culture showed tuberculosis.

Case 2

A 30-year-old man had fever and obscure abdominal pain for two months with no clinically palpable abdominal mass.

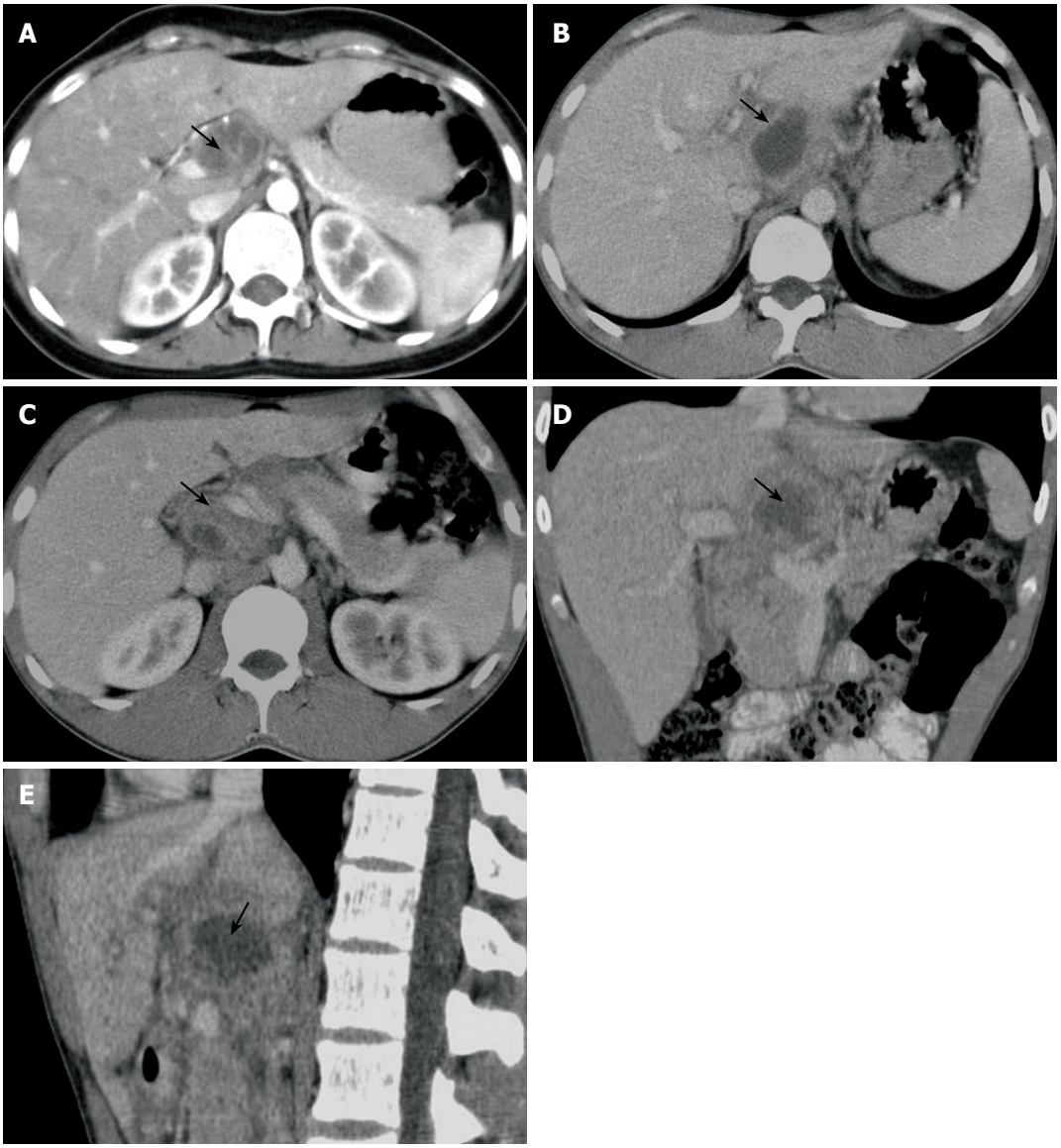

Figure 1 A 24-year-old man with tuberculous abscess in the hepatoduodenal ligament. **A**: Axial CT image (arterial phase) showing a low dense abscess measuring approximately 3.9 cm × 2.8 cm with a slightly contrast-enhanced wall in hepatoduodenal ligament (arrow); **B**: Axial CT image (venous phase) showing a low dense abscess with a contrast-enhanced wall in hepatoduodenal ligament (arrow); **C**: Axial CT image showing enlarged lymph nodes with peripheral and homogenerous enhancement in the portacaval space (arrow); **D**: Coronal CT image showing a low dense abscess with a contrast-enhanced wall in hepatoduodenal ligament (arrow); **E**: Sagittal CT image showing a low dense abscess with a contrast-enhanced wall in hepatoduodenal ligament (arrow).

Contrast-enhanced CT was performed on a spiral CT scanner (Elscint HeliCAT Flash). The image protocol consisted of venous phase. Power injector (MCT Plus, Meddred, Pittsburg) was used. Contrast material was Ultravist (Schering Germany, 300 mgI/mL). The range of CT scan covered the upper and middle abdomen. CT parameters were 120 KV, 250 mA, pitch 1. CT scan was performed with intravenous contrast material administered as a bolus. Oral contrast material (1.2% angiografin) was administrated. Images were viewed at a window width setting of 400 HU and a window level setting of 50 HU.

Contrast-enhanced CT showed a less dense mass with thick enhanced irregular walls in the hepatoduodenal ligament. The mass measured 4.1 cm × 2.7 cm with its margin poorly defined. The interface between the mass and surrounding organs was not clear. Gas collection was not shown. Enlarged lymph nodes were detected in the portacaval space, gastrohepatic ligament, which had peripheral enhancement (Figure 2A and B).

Operation on abdominal region was performed. Macroscopic pathological examination showed that the mass was tightly adhered to the hepatoduodenal ligament. Enlarged lymph nodes were found. Twenty mL of pus was aspirated from the mass. Microscopic examination revealed that the mass was tuberculosis.

Both patients gave their written informed consent.

DISCUSSION

Tuberculosis demonstrates a variety of clinical and radiologic features depending on the involved organ and has a known propensity for dissemination from its primary site. Thus, tuberculosis can mimic a number of other disease entities, and it is important to be familiar with the various radiologic features of tuberculosis to establish its diagnosis early and accurately[7].

Lymphadenopathy is the most common manifestation of abdominal tuberculosis[5,6]. Mesenteric, omental and peripancreatic lymph nodes are most commonly involved[5]. Abdominal tuberculosis may be transmitted by three major routes. The first is ingestion of material infected with tubercle bacilli which are carried from a lesion in the intestinal submucosal layer to the lymph nodes draining that segment of the bowel. Drainage is usually from lymphatics

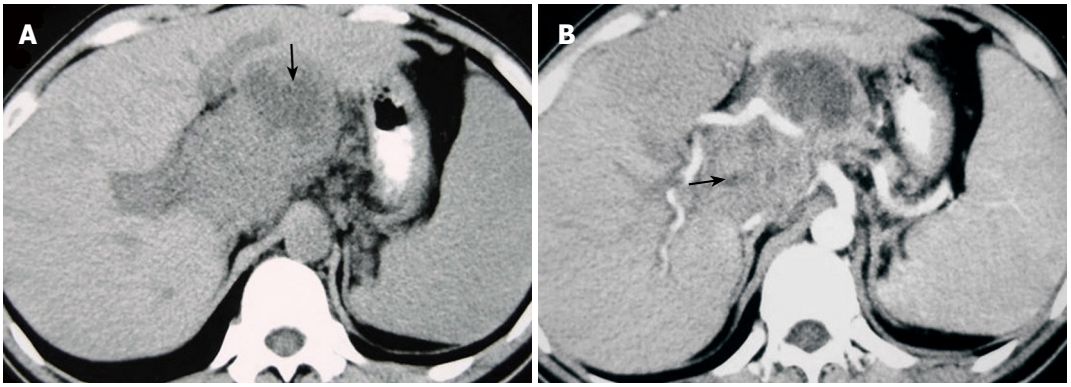

Figure 2 A 30-year-old man with a tuberculous abscess in the hepatoduodenal ligament. **A**: Axial plain CT image showing a low dense abscess measuring approximately 4.1 cm × 2.7 cm in hepatoduodenal ligament (arrow); **B**: Axial contrast-enhanced CT image showing a low dense abscess with a contrast-enhanced wall and enlarged lymph nodes in hepatoduodenal ligament (arrow).

of the ileocecum, jejunum, ileum, and right side of colon to the peripancreatic and superior mesenteric lymph nodes. The second route of transmission is hematogenous spread. Bacteria are disseminated from a distant site of infection, usually the lungs, to the abdominal lymphatic system. Because this process is systemic, it may cause infection of mesenteric lymph nodes. In the third route of transmission, infection can spread directly to the abdominal lymph nodes from the serosa of adjacent infected structures[5]. In this study, two patients had non-disseminated tuberculosis and CT showed enlarged lymph nodes in the portacaval space, gastrohepatic ligament, peripancreatic and upper paraaortic region. So, the anatomic distribution of this disease closely parallels to the route of tuberculous infection.

The nodes are usually large and multiple, and most commonly demonstrate peripheral enhancement with central areas of low attenuation on contrast-enhanced CT images[5]. Pathologic findings from surgically obtained specimens of tuberculous lymphadenopathy indicate that caseation or liquefactive substances at the center of enlarged lymph nodes have a low attenuation that presumably results from insufficient blood supply, whereas peripheral inflammatory lymphatic tissue has a higher attenuation on enhanced CT that results from the preserved blood supply[8]. In this study, both cases were accompanied with enlarged lymph nodes, suggesting that the tuberculous abscess may be due to the coalescence of the involved lymph nodes.

Cystic lesions, including pseudocysts, necrotic tumors, and cysts of the pancreas and/or adjacent organs, must be differentiated from tuberculous abscess in the hepatoduodenal ligament. Pseudocyst is a unilocular, round mass with a uniform wall, and can be found in patients with clinical and laboratory evidence of pancreatitis. A well-defined rind suggests a pseudocyst or abscess, and gas bubbles suggest an abscess. CT demonstrates small calcification and fat in the teratomas located in the hepatoduodenal ligament area, and its border is round and sharp[9-11]. CT of extension of gastrointestinal stromal tumors displays extraluminal growth, inhomogeneous enhancement, absence of calcifications and lymph node metastases[12]. Congenital cysts (duplication, mesenteric, omental, or choledochal) may be localized to the hepatoduodenal ligament area. However, the history, clinical findings and the absence of enlarged lymph nodes do not suggest tuberculosis[11]. Serous and mucinous cystadenomas are encapsulated and lobulated masses, showing marked contrast-enhancement of the solid portion. Cystadenocarcinoma is seen as a multilobular, septate, thick-walled cyst or cystic neoplasm with multiple low-density areas. Dilatation of the main pancreatic duct may be seen[10]. The radiologic pattern of tuberculous lymphadenitis can also be seen with lymphoma[7]. The enhancement patterns of untreated lymphomas are homogeneous. In patients with lymphoma who have undergone therapy, central low attenuation may be found within nodes, simulating tuberculous lymphadenopathy. So, it is important to know the history[13-16].

It was reported that tuberculous lymphadenopathy has the following clinical characteristics[17]: (1) some patients have a history of TB and most of them come from areas with a high prevalence of active tuberculosis; (2) patients often suffer from epigastric pain, fever and weight loss; (3) ultrasound and CT scan show enlarged nodules, sometimes with focal calcification. In this study, a patient had weight loss, fatigability, night sweats, and obscure abdominal pains, while the other patient had fever and obscure abdominal pains. Both patients had no clinically palpable abdominal mass.

In summary, tuberculous abscess in the hepatoduodenal ligament is a less dense mass with thick enhanced irregular walls, its margin is poorly-defined, and the interface between the mass and around organs is not clear. It is important to show the peripheral enhanced lymph nodes for its early and accurate diagnosis.

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LETTERS TO THE EDITOR

Occult hepatitis C virus infection is more common than hepatitis B infection in maintenance hemodialysis patients

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Abstract

Patients of end stage renal disease on maintenance hemodialysis were enrolled to study the prevalence of occult and dual hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and non-occult hepatitis B and C virus infection. One hundred and two patients were enrolled. Thirty patients had HCV infection, three of them were positive in anti-HCV. So, 27 (90%) of HCV-positive patients had occult HCV infection. Eleven (11%) patients had HBV infection. Five patients were positive in anti-HBc or HBV-DNA, but negative in HBsAg (occult HBV infection). Three (3%) patients had dual HBV and HCV infection. None of the patients showed changes in viral markers during the follow-up of 8 mo on average (1-12 mo).

Key words: Occult hepatitis C; Hepatitis B; Maintenance hemodialysis

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TO THE EDITOR

We read with interest the article "Hepatitis B viral infection in maintenance hemodialysis patients: A three-year follow-up" by Cao *et al* in 13(45): 6037-6040, 2007, *World Journal of Gastroenterology*[1]. We agree that the hepatitis B vaccination and regular surveillance for hepatitis B virus (HBV) infection has reduced the spread of HBV in the dialysis population. The prevalence of hepatitis C virus (HCV) infection in hemodialysis (HD) patients is high and ranges from 2% to 60% between countries and among dialysis units[2]. The prevalence of HBV and HCV occult and dual infection[3,4] in hemodialysis patients has been variably reported.

We prospectively studied consecutive patients of end stage renal disease (ESRD) on maintenance of HD from June 2006 to June 2007 for prevalence of occult and dual hepatitis B and C virus infection and non-occult hepatitis B and C virus infection. Occult hepatitis C infection was defined as anti-HCV negative and HCV-RNA positive by polymerase chain reaction[3,5]. All patients underwent tests of hemoglobin, urea, creatinine,bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The viral markers done were hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B envelope antigen (HBeAg), antibody to hepatitis Be antigen (anti-HBe), antibody to hepatitis C virus (anti-HCV) by enzyme linked immunoassay (ELISA) and qualitatively hepatitis B virus DNA (HBV DNA) and hepatitis C virus RNA (HCV RNA) by polymerase chain reaction.

The demographic, clinical features, biochemical parameters, etiology, history of blood transfusion and time on hemodialysis are described in Table 1. One hundred and two patients were enrolled. The mean age was 41.4 years (range 17-70 years) with a male: female ratio of 68:34. The clinical presentations were generalized swelling 36 (36%), decreased urine output 34 (34%), breathlessness 30 (30%), hypertension 24 (24%) and altered sensorium in 8 patients. The mean hemoglobin, urea, creatinine, bilirubin, AST and ALT were 76.5 mg/L (33-122 mg/L) 184.3 mg/L (84-322 mg/L), 10.8 mg/L (4-23 mg/L), 0.6 mg/L (0.4-0.8 mg/L), 53.5 unit/L (26-188 unit/L) and 38.6 unit/L (16-209 unit/L). Thirty-four patients had histories of blood transfusion.

Among HD patients with HCV infection, serum ALT was elevated in 10 HCV-RNA positive patients, but normal in all the anti-HCV positive patients. Thirty (30%) patients had HCV infection, three them had anti-HCV positivity. So, twenty-seven (90%) of HCV-positive patients had occult HCV infection.

Eleven (11%) patients had HBV infection. Five patients

Table 1 Demographics, clinical and biochemical parameters of patients on maintenance hemodialysis

Male:Female	68:34
Age (yr)[1]	41.4 (17-70)
Clinical features, cases (%)	
Generalised swelling	36 (36)
Oliguria	34 (34)
Breathlessness	30 (30)
Hypertension	24 (24)
Altered sensorium	8 (8)
Laboratory parameters[1]	
Hemoglobin (mg/L)	76.5 (33-122)
Urea (mg/L)	184.3 (84-322)
Creatinine (mg/L)	10.8 (4-23)
Bilirubin (mg/L)	0.6 (0.4-0.8)
Aspartate aminotransferase (unit/L)	53.5 (26-188)
Alanine aminotransferase (unit/L)	38.6 (16-209)
History of blood transfusion, cases (%)	34 (34)
Past history of jaundice, cases (%)	4 (4)
Etiology, cases (%)	
Chronic glomerulonephritis	44 (44)
Chronic interstitial nephritis	20 (20)
Diabetes mellitus	20 (20)
Polycystic kidney disease	5 (5)
Glomerulopathy, unknown	13 (13)
Time on hemodialysis (mo)[1]	34 (12-60)

[1]Mean (range).

were positive in anti-HBc or HBV-DNA but negative in HBs Ag (occult HBV infection). Rai *et al* reported 12.2% occult HBV infection and 10.3% occult HCV infection in human immunodeficiency virus patients[5]. Goral *et al* reported that occult HBV infection was not high in chronic HCV infected patients on HD[6].

Three (3%) patients had dual HBV and HCV infection. Reddy *et al* found dual infection in 3.7% of patients on HD[4]. None of the viral markers were positive in 20 patients. Four patients had past histories of jaundice, three of them had HBV infection and one was positive in HCV-RNA.

Thirty patients with positive viral markers had histories of blood transfusion ranging from 1-6 units. Agarwal *et al*[7] showed in their studies in 208 ESRD patients with past histories of jaundice and the number of blood transfusion was significantly higher in HCV positive patients than in HCV negative patients. In our study, blood transfusion history was present in most of the patients (n = 26) with HCV infection. Two patients had past histories of jaundice.

On follow-up of mean 8 mo (1-12 mo), none of the patients showed change in viral markers. Twelve patients died of cardiac arrhythmias due to hyperkalemia, fluid overload due to inadequate dialysis and sepsis. In our study, the development of cirrhosis, hepatocellular carcinoma and decompensation of liver function were not observed in HCV and HBV infected patients.

Yakaryilmaz *et al* in their group of 188 ESRD patients on maintenance of HD showed 28.7% had both occult and non-occult forms of HCV infection which was more common than HBV (19.7%) infection[3].

HBV infection was present in 11% of patients on maintenance HD possibly due to a higher percentage (44%) of patients having protective anti-HBs titres. In the previous studies, HBV DNA positive hemodialysis patients had a significantly lower prevalence of past HBV vaccination and lower anti-HBs titres in serum than HBV DNA-negative patients of the same group[8]. Nijhawan *et al* did the screening of 69 330 subjects for HBsAg and found that prevalence of HBsAg in replacement donors was 3.1% and 2.1% in healthy voluntary donors[9]. So, HBV infection is relatively higher in patients on HD.

So, HCV-RNA is recommended in patients on HD and now has been included in our screening program prior to renal transplantation. HBV vaccination of HD patients is an effective way of limiting the risk of transmission of HBV infection to patients on hemodialysis.

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ANNOUNCEMENT

The article published in *WJG* 2005; 11(19): 2975-2980 plagiarized an article previously published in *Journal of Gastroenterology* 2004; 39(2): 104-112

From the editor

On March 5, 2008, we received an E-mail reporting a case of plagiarism. After our evaluation we were able to confirm this claim. The details are noted below. In fact, we have published an article[1] at http://www.wjgnet.com/1007-9327/13/2019.asp [*World J Gastroenterol* 2007 April 14;13 (14): 2019], which announced the attitudes of *World Journal of Gastroenterology* towards such a behavior. Now we are exposing it again to draw more attention.

The article published in *WJG* 2005; 11(19): 2975-2980 plagiarized an article previously published in *Journal of Gastroenterology* 2004; 39(2): 104-112. *World J Gastroenterol* 2008; 14(14): 2290-2291 Available from: URL: http://www.wjgnet.com/1007-9327/14/2290.asp DOI: http://dx.doi.org/10.3748/wjg.14.2290

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Table 3 Correlation of pathological factors and expression of syndecan-1

	Syndecan-1 expression		P
	Positive (n = 80)	Negative (n = 48)	
Histology			
Solely differentiated type	66	36	<0.05
Complextype	14	24	<0.05
Lymphatic and / or venous invasion	28	13	NS
Lymph node metastasis	12	9	NS
IM with G-type	2	6	<0.05

G-type, gastric type; NS, not significant. 1Evaluated in 61 surgically resected specimens with submucosal invasion. 2Evaluated in 15 cases with positive for IM.

Table 4 Correlation of pathological factors and E-cadherin

	E-cadherin expression		P
	Positive (n = 73)	Abnormal (n = 43)	
Histology			
Solely differentiated type	57	39	NS
Complextype	20	14	NS
Lymphatic and / or venous invasion	23	18	NS
Lymph node metastasis	7	13	<0.05
IM with G-type	4	4	NS

G-type, gastric type; NS, not significant. 1Evaluated in 61 surgically resected specimens with submucosal invasion. 2Evaluated in 15 cases with positive for IM.

none (0%) of N-type cancers (Table 4). Therefore, G-type IM was accompanied significantly more often by G-type cancers than by non G-type, including O-type and CI-type cancers ($P<0.05$; Table 4). In addition, G-type IM was observed significantly more often in cancers with negative expression of syndecan-1 (75%) than in those with positive expression (25%; $P<0.05$), although there was no such relation for E-cadherin expression (Table 4). Reduction or loss of syndecan-1 expression and abnormal expression of E-cadherin were not seen in foveolar epithelium.

DISCUSSION

Syndecans are a family of cell-surface transmembrane heparan sulfate proteoglycans. The sugar side chains of syndecan are structurally related to heparin and have functional cytoplasmic and extracellular domains that are thought to participate in both cell-cell and cell-extracellular matrix adhesion[20-22]. Syndecan-1 is expressed not only in epithelial tissue but also in fibroblasts and plasma cells[20-22]. Studies of the role of syndecan-1 in malignant transformation have revealed that syndecan-1 expression is associated with the maintenance of epithelial morphology and inhibition of invasion[23-26]. Reduced expression of syndecan-1 was associated with malignant transformation in hepatocellular carcinoma[26] and with poor prognosis in colorectal carcinoma[28]. Furthermore, the expression of syndecan-1 was augmented during epithelial regeneration and reintegument in the stomach[29]. Syndecan-1 plays an important role in cell-cell adhesion, and many studies have examined the role of syndecan-1 in oncogenesis[30-34].

A number of authors have reported about an association between E-cadherin expression and gastric cancer[35-37]. However, there are few studies evaluating syndecan-1 in comparison with other adherent molecules, including E-cadherin, in other malignancies[23-26]. And there are no data that compare syndecan-1 and E-cadherin expression in each cellular phenotype in early gastric cancers. In the present study, we found that syndecan-1 expression was significantly reduced in G-type cancers and G-type IM compared with the other phenotypes of cancers and IM. Furthermore, G-type IM was accompanied significantly more often by G-type cancers than by O-type and CI-type cancers. These data suggest that the reduction of syndecan-1 may play a role in the growth of both G-type gastric cancer and G-type IM. G-type cancers are considered to have a more aggressive nature when compared with cancers of other cellular phenotypes[38,39]. Therefore, the altered expression of syndecan-1 in early-stage gastric cancers may alter the biological behavior of the transformed epithelial cells and affect the invasive characteristics. In addition, assessment of the expression level of syndecan-1 may serve as a novel biomarker for predicting the malignant potential of cancers.

Human gastric foveolar epithelium, IM, and most DGC cells expressed syndecan-1 protein and mRNA, as reported previously[40]. Syndecan-1 was mainly localized at the basolateral surfaces of the cells, implying that it participates in cell-cell and cell-extracellular matrix adhesion[41-43]. The expression of syndecan-1 was lost or reduced in G-type cancers, which were significantly associated with complex type cancers, comprised predominantly of DGCs and partly of poorly differentiated adenocarcinoma or signet-ring cell carcinoma. It is reported that DGCs with gastric phenotype tend to change to undifferentiated-type adenocarcinoma[44]. Therefore, the reduction of syndecan-1 might affect dedifferentiation, which appears as the loss of tumor glandular formation. Previous report indicates that the detachment of invading cancer cells from the nests was more frequently observed in syndecan-1-negative colorectal cancers[28]. As for E-cadherin, however, its expression did not relate to cellular phenotype or to histologically complex type cancer. A report by Kowbi *et al*[45], showed that G-type cancer frequently demonstrated the abnormal expression of E-cadherin. Risk *et al*[46], speculated that a reduction or loss of E-cadherin expression, with the concomitant acquisition of a morphologically diffuse growth pattern, might be responsible for the development of poorly differentiated and/or dedifferentiated cells in intestinal type gastric cancer (which corresponds to our complex type cancer). The reasons for the discrepancy in these results are considered to be the differences of patient numbers examined and of the incidence of complex type in G-type cancers. Another explanation might be a defect in the catenin part of E-cadherin/catenin complex[45]. Day *et al*[35], reported that a greater reduction of syndecan-1 expression than of E-cadherin expression was seen in the transition from moderate to severe dysplasia in colon tumors. This result suggests that the changes in the expression of syndecan-1 probably occur before those in E-cadherin, perhaps influencing the expression of the latter adhesion molecule[35]. Thus, our data are in agreement with their results. IM is generally known to be a precursor of DGC[47,48]. In this study, we

Figure 1 Snapshot of manuscript (6 pages total) published by *WJG*. The yellow parts were copied directly from the manuscript published by *Journal of Gastroenterology*.

ANALYSIS OF PLAGIARIZING PUBLICATION OF MANUSCRIPT

Both manuscripts focus on the expression of Syndecan-1 and E-cadherin in differentiated type of gastric cancer, and most parts of the article published by *WJG* are exact copies of the manuscript published by *Journal of*

Gastroenterology without acknowledgment and citations including the same sentences, words and even punctuation marks. The yellow highlighted parts in Figure 1 show the same parts in the article published by *WJG*.

Based on this analysis, *WJG* considers this a typical case of plagiarism, and is prepared to take strict, appropriate action against such behavior. Authors from the plagiarizer's group are not accepted to submit their papers in the future.

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Meetings

Events Calendar 2008-2009

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www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai - Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas
Email: robert.giuli@oeso.org

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008
June 4-7, Helsinki, Finland

The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008
June 6-8, Prague, Czech Republic
3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
Email: meetings@imedex.com

June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 25-28, Barcelona, Spain
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E-mail: office@epc-iap2008.org
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June 26-28, Bratislava, Slovakia
5th Central European Gastroenterology Meeting
www.ceurgem2008.cz
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III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
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Falk Symposium 166:
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www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
Falk Symposium 167:
Liver Under Constant Attack - From Fat to Viruses
September 24-27, Nantes, France
Third Annual Meeting
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October 8-11, Istanbul, Turkey
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October 18-22, Vienna, Austria
16th United European Gastroenterology Week
www.negf.org
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October 31-November 4, Moscone West Convention Center, San Francisco, CA
59th AASLD Annual Meeting and Postgraduate Course
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Information: www.aasld.org

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Email: ngm2008@mci-group.com
www.ngm2008.com

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Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

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E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
Digestive Disease Week 2009

November 21-25, London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
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