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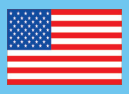

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WJG World Journal of Gastroenterology

Contents

Weekly Volume 16 Number 15 April 21, 2010

EDITORIAL

1811 Role of monocytes and macrophages in experimental and human acute liver failure
Possamai LA, Antoniades CG, Anstee QM, Quaglia A, Vergani D, Thursz M, Wendon J

1820 Modifier-concept of colorectal carcinogenesis: Lipidomics as a technical tool in pathway analysis
Gassler N, Klaus C, Kaemmerer E, Reinartz A

OBSERVATION

1828 Risk factors in familial forms of celiac disease
Freeman HJ

REVIEW

1832 Chemokines and hepatocellular carcinoma
Huang F, Geng XP

ORIGINAL ARTICLE

1837 Role of HSP-90 for increased nNOS-mediated vasodilation in mesenteric arteries in portal hypertension
Moleda L, Jurzik L, Froh M, Gäbele E, Hellerbrand C, Straub RH, Schölmerich J, Wiest R

1845 Different profiles of cytokine expression during mild and severe acute pancreatitis
Dambrauskas Z, Giese N, Gulbinas A, Giese T, Berberat PO, Pundzius J, Barauskas G, Friess H

1854 Insulin-like growth factor-I receptor in proliferation and motility of pancreatic cancer
Tomizawa M, Shinozaki F, Sugiyama T, Yamamoto S, Sueishi M, Yoshida T

1859 Effect of S1P5 on proliferation and migration of human esophageal cancer cells
Hu WM, Li L, Jing BQ, Zhao YS, Wang CL, Feng L, Xie YE

BRIEF ARTICLE

1867 Incidence and risk factors for the development of anemia following gastric bypass surgery
Avgerinos DV, Llaguna OH, Seigerman M, Lefkowitz AJ, Leitman IM

1871 Ischemic preconditioning-induced hyperperfusion correlates with hepatoprotection after liver resection
Heizmann O, Meimarakis G, Volk A, Matz D, Oertli D, Schauer RJ

Contents

World Journal of Gastroenterology
Volume 16 Number 15 April 21, 2010

1879 Comparative analysis of clinicopathological correlations of cyclooxygenase-2 expression in resectable pancreatic cancer
Hermanova M, Karasek P, Tomasek J, Lenz J, Jarkovsky J, Dite P

1885 Non-small-bowel lesions encountered during double-balloon enteroscopy performed for obscure gastrointestinal bleeding
Tee HP, Kaffes AJ

1890 Prognosis of cancer with branch duct type IPMN of the pancreas
Ikeuchi N, Itoi T, Sofuni A, Itokawa F, Tsuchiya T, Kurihara T, Ishii K, Tsuji S, Umeda J, Moriyasu F, Tsuchida A, Kasuya K

1896 Gender differences of low-dose aspirin-associated gastroduodenal ulcer in Japanese patients
Okada K, Inamori M, Imajyo K, Chiba H, Nonaka T, Shiba T, Sakaguchi T, Atsukawa K, Takahashi H, Hoshino E, Nakajima A

1901 Chemotherapy with laparoscope-assisted continuous circulatory hyperthermic intraperitoneal perfusion for malignant ascites
Ba MC, Cui SZ, Lin SQ, Tang YQ, Wu YB, Wang B, Zhang XL

1908 Remission induction and maintenance effect of probiotics on ulcerative colitis: A meta-analysis
Sang LX, Chang B, Zhang WL, Wu XM, Li XH, Jiang M

1916 Natural taurine promotes apoptosis of human hepatic stellate cells in proteomics analysis
Deng X, Liang J, Lin ZX, Wu FS, Zhang YP, Zhang ZW

CASE REPORT

1924 Cystic fibrosis and Crohn's disease: Successful treatment and long term remission with infliximab
Vincenzi F, Bizzarri B, Ghiselli A, de'Angelis N, Fornaroli F, de'Angelis GL

1928 Use of biological meshes for abdominal wall reconstruction in highly contaminated fields
Cavallaro A, Lo Menzo E, Di Vita M, Zanghì A, Cavallaro V, Veroux PF, Cappellani A

1934 Gastric adenomyoma presenting as melena: A case report and literature review
Zhu HN, Yu JP, Luo J, Jiang YH, Li JQ, Sun WY

LETTERS TO THE EDITOR

1937 Hepatoprotective effects of antioxidants in chronic hepatitis C
Moreno-Otero R, Trapero-Marugán M

Contents

World Journal of Gastroenterology
Volume 16 Number 15 April 21, 2010

ACKNOWLEDGMENTS I Acknowledgments to reviewers of *World Journal of Gastroenterology*

APPENDIX I Meetings

I-IV Instructions to authors

AIM AND SCOPE

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EDITORIAL

Role of monocytes and macrophages in experimental and human acute liver failure

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Abstract

Acute liver failure (ALF) is a devastating clinical syndrome characterised by progressive encephalopathy, coagulopathy, and circulatory dysfunction, which commonly leads to multiorgan failure and death. Central to the pathogenesis of ALF is activation of the immune system with mobilisation of cellular effectors and massive production of cytokines. As key components of the innate immune system, monocytes and macrophages are postulated to play a central role in the initiation, progression and resolution of ALF. ALF in humans follows a rapidly progressive clinical course that poses inherent difficulties in delineating the role of these pivotal immune cells. Therefore, a number of experimental models have been used to study the pathogenesis of ALF. Here we consider the evidence from experimental and human studies of ALF on the role of monocytes and macrophages in acute hepatic injury and the ensuing extrahepatic manifestations, including functional monocyte deactivation and multiple organ failure.

INTRODUCTION

Acute liver failure (ALF) is characterised by overwhelming hepatic injury with failure of hepatocyte function, resulting in a devastating clinical syndrome of coagulopathy, encephalopathy, and circulatory dysfunction[1,2]. Phenotypically, there are striking similarities between septic shock and ALF. Both conditions are characterised by activation of a systemic inflammatory response syndrome (SIRS), resulting in circulatory dysfunction with systemic vasodilatation and refractory multiorgan failure[3]. ALF, like septic shock, is associated with an overwhelming activation of the immune response, including the production of inflammatory cytokines and mobilisation of cellular components of the immune system. Monocytes and macrophages are key orchestrators of the innate immune system and are postulated to play a pivotal role in the initiation, propagation and resolution of ALF.

The paucity of effective clinical treatments for ALF, except for supportive care and liver transplantation, re-

flects our poor understanding of the pathogenesis of ALF. Clinical trials and studies on patients with ALF are limited by small numbers of subjects with heterogeneous causes of ALF and are confounded by inherent difficulties in studying patients with rapidly progressive pathology. There are a number of animal models of acute liver injury commonly used to study ALF: murine models of acetaminophen (APAP) toxicity, concanavalin-A (Con-A) T-cell mediated hepatitis, carbon tetrachloride (CCl_4)-induced injury, and other models including galactosamine/lipopolysaccharide (Gal/LPS) (for a review[4]). Unfortunately, these are models of acute liver injury and cannot truly reflect the human syndrome of ALF.

In this article we consider the role of macrophages, monocytes, and the inflammatory cytokines they produce, in the progression of acute liver failure in human and experimental models. We consider monocyte/macrophage function in the hepatic and systemic compartments, including their role in initial injury, the process of recruitment of monocytes to the injured liver and the possible influence of hepatic inflammatory events on the functional monocyte deactivation encountered in ALF.

RECRUITMENT OF MONOCYTES TO THE INJURED LIVER

Expansion of hepatic macrophage populations

Following an acute hepatic insult, there is a rapid and marked increase in the number of inflammatory cells within the liver. Recent studies have shed light on the process by which circulating monocytes are recruited to the injured liver, where, due to their plasticity, macrophages are implicated in both tissue destructive and reparatory processes of the inflammatory response.

Karlmark *et al*[5] demonstrated, in a murine model of acute CCl_4 hepatotoxicity, that a sub-population of infiltrating macrophages account for 10%-12% of total hepatic cells 24-48 h after the onset of liver injury. This massive recruitment of macrophages and their contribution to evolving acute liver failure have attracted much interest recently. Two distinct populations of macrophage have been described in both APAP and acute CCl_4 models of hepatotoxicity[5,6]. The first is a $CD11b^+$ $F4/80^-$ population that resembles the sub-group of circulating monocytes that are the origin of stable populations of tissue macrophages. This population shows only a modest increase in number following acute liver injury by CCl_4. The second population is $CD11b^+$ $F4/80^+$ and this resembles the subset of circulating monocytes from which activated macrophages in inflammatory conditions are thought to be derived. It is this population that shows marked expansion in number following acute hepatic injury[5].

Holt *et al*[6] 2008, in an APAP model of hepatoxicity, showed that these "inflammatory macrophages" were derived from circulating monocytes rather than from proliferation of the resident Kupffer cell (KC) population. They noted preservation of the macrophage response to acute APAP toxicity when the KC population was depleted prior to APAP challenge. Conversely, when circulating monocytes were depleted by bone marrow irradiation prior to APAP, the number of intrahepatic macrophages seen after tissue injury was diminished.

The role of chemokine: Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family, which acts on the chemokine (C-C motif) receptor 2 (CCR2) that is expressed on macrophages, monocytes, and to a lesser extent, T and NK cells[7]. Though initially described as a monocyte chemokine it also plays a role in the recruitment of NK cells and T cells in a wide range of inflammatory conditions[8]. An early increase in hepatic expression of MCP-1 and other chemokines is a consistent feature of murine models of ALF. An increase in MCP-1 mRNA or total hepatic levels of MCP-1 have been reported within 12 h of liver insult in APAP, Con-A, Gal/LPS, and CCl_4 models of acute liver failure[5,9-12]. The origin of hepatic MCP-1 in ALF is thought to be both resident KCs and injured hepatocytes[13].

MCP-1/CCR2 interaction in animal models

Experiments using CCR2 -/- knockout mice suggest that functional CCR2 is necessary for egress of $F4/80^+$ monocytes from the bone marrow. In the acute liver injury model induced by CCl_4, CCR2 -/- mice demonstrated a decreased number of hepatic and serum $CD11b^+$ $F4/80^+$ cells following liver injury, but an increase in this cellular fraction in bone marrow when compared with wild-type mice[10]. When this population of monocytes was adoptively transferred into the circulation of CCR2 -/- mice they were able to traffic unhindered into the injured liver[5]. Similarly to the liver injury model, CCR2 has been shown to be essential for monocyte mobilisation from the bone marrow, but not for efflux into inflamed tissues, such as in murine *CMV*, *L. monocytogenes,* and urinary tract infection models[8,14,15].

This evidence suggests that hepatic-derived MCP-1 is able to stimulate the expansion of a bone marrow population of $CD11b^+$ $F4/80^+$ monocytes, and CCR2 is necessary for these cells to exit the bone marrow, but not essential for influx into the injured liver[5].

MCP-1 in human ALF

In human ALF, there is also evidence of early upregulation of hepatic chemokines. Leifeld *et al*[9] demonstrated serum MCP-1 levels that were, on average, five times higher in patients with ALF when compared to patients with self-limiting acute hepatitis. MCP-1 levels were also significantly elevated above healthy control populations and those with chronic liver disease. There was a trend suggesting that higher serum MCP-1 levels were associated with poor outcome (death or transplantation) from ALF. Upregulation of chemokines, including MCP-1, was demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry. The latter technique showed that MCP-1 expression was not limited to inflammatory cells only, but was also de-

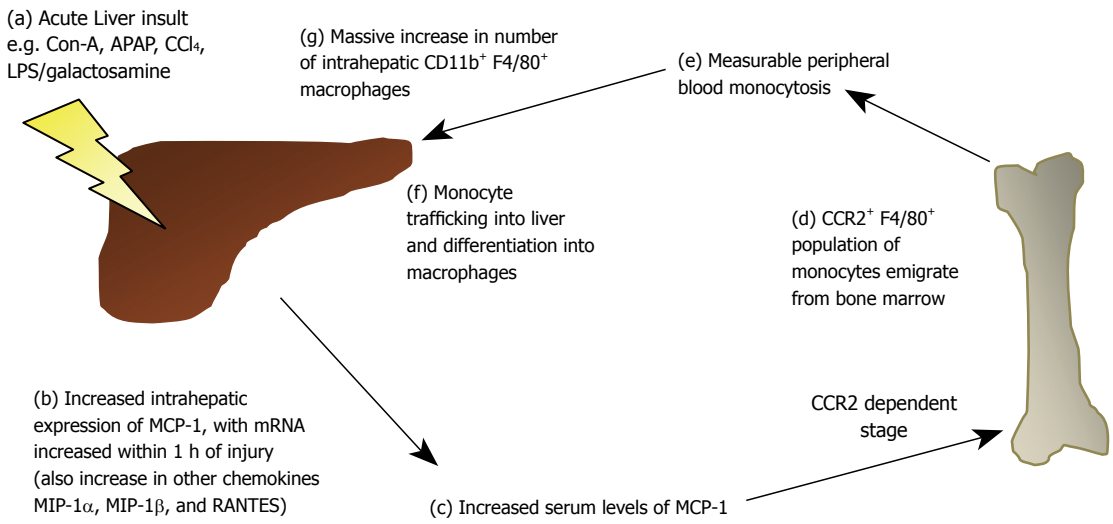

Figure 1 Summary of monocyte/macrophage recruitment to the liver following an acute hepatic insult. Con-A: Concanavalin-A; APAP: Acetaminophen induced hepatotoxicity; CCl_4: Carbon tetrachloride; LPS: Lipopolysaccharide; CCR2: Chemokine (C-C motif) receptor 2; MCP-1: Monocyte chemoattractant protein-1; MIP: Macrophage inflammatory protein; RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted, abbreviated (CCL5).

tected in hepatocytes, biliary epithelial cells, and sinusoidal endothelial cells (SEC) in human ALF.

These findings have been replicated in patients with acute severe alcoholic hepatitis with clinical liver failure. MCP-1 is seen to be upregulated throughout the liver, with expression observed in hepatocytes and sinusoidal endothelial cells[16]. Conversely, in control patients with stable alcoholic liver disease, MCP-1 expression is limited to non-parenchymal cells.

In paediatric patients with acute acetaminophen hepatotoxicity, serum MCP-1 levels showed a strong correlation with clinical parameters of severe liver injury, including elevated levels in patients in whom treatment with N-acetyl cysteine was delayed[17].

Thus evidence from both murine and human studies suggests that early release of chemokines from injured hepatocytes, SECs and KCs in ALF is responsible for the mobilisation and subsequent trafficking of a population of activated monocytes/macrophages to the liver from the bone marrow (summarised in Figure 1).

THE ROLE OF HEPATIC MACROPHAGES IN THE PROGRESSION OF ACUTE LIVER FAILURE

Having established that macrophages are recruited to the liver in acute injury, the challenge that remains is determining what role these cells play in liver damage or repair.

Two early studies in animal models of APAP-induced hepatotoxicity suggested that depletion of macrophage numbers with gadolinium chloride or dextran sulphate prior to APAP dosing offered protection from liver damage[18,19]. However, neither of these studies confirmed successful depletion of intrahepatic macrophages, and in one study, the number of intrahepatic F4/80$^+$ cells post-APAP treatment was actually increased, suggesting their prior treatment with KC depleting agents had not prevented an expansion in the macrophage population following the toxic insult[19]. Subsequent studies have used the more effective technique of macrophage depletion with liposome/clodronate. An APAP model of acute liver failure has shown macrophage depletion increases sensitivity to toxic injury, as measured at 8 and 24 h post-APAP alanine transaminase (ALT) values[20]. Conversely, in an acute CCl_4 model, macrophage depletion was reported to have no effect on ALT levels four and 24 h after toxin administration[5].

Prevention of liver injury-induced macrophage population expansion by inhibition of the MCP-1/CCR2 interaction has been demonstrated in a number of studies using CCR2 knockout mice. As described above, these animals show a marked reduction in intrahepatic macrophages following liver injury compared with wild-type (WT) mice, due to failure of monocyte recruitment from bone marrow. Two studies in CCR2 -/- mice treated with toxic doses of APAP have shown ALT levels in the first 24 h comparable to WT controls[6,13]. Histological evidence of liver damage was also comparable between CCR2 -/- and WT mice at 24 h in both studies. At later time points, the two studies showed divergent results, with Dambach *et al*[13] showing less inflammation in CCR2 -/- mice. However, Holt *et al*[6] described CCR2 -/- mice showing persisting necrosis and ongoing inflammation at 72 h, whereas the wild-type animals showed almost complete histological resolution. Hogaboam *et al*[12] used a similar model of acute APAP toxicity in CCR2 -/- mice and results seemed to concur with Holt's study, showing a worsening of liver injury by ALT values and histology in the knockout animals at all time points when compared to WT controls.

Overall current evidence in murine models is conflicting (Table 1). It appears that depletion of resident KCs prior to, and at the onset of, liver injury reduces the extent of liver damage. Kupffer cells are partly responsible for the initial pro-inflammatory response to injury with cytokine production and the recruitment of multiple in-

Table 1 Literature summary on the effect of hepatic macrophage depletion on the extent of liver injury in experimental models of ALF

Study	Model	Method of macrophage depletion/inhibition	Affect on severity of acute liver injury	Conclusion
Laskin *et al*[18], 1995	APAP (rat)	Gadolinium chloride/ dextran sulphate	Decreased ALT at 24 h in treated groups; Decreased necrosis	Macrophage depletion was protective
Michael *et al*[19], 1999	APAP (mouse)	Gadolinium chloride/ dextran sulphate	Decreased ALT at 8 h in treated groups	Macrophage depletion was protective
Hogaboam *et al*[12], 2000	APAP (mouse)	CCR2 -/-	ALT at 24 and 48 h, hepatic necrosis and TUNEL staining all increased in KO; Increase in IFN-γ and TNF-α	CCR2 KO - macrophage depletion worsened liver injury
Dambach *et al*[13], 2002	APAP (mouse)	CCR2 -/-	ALT levels similar in WT and KO mice; Histologically KO mice showed less inflammation at 72 h	CCR2 KO - macrophage depletion, caused less inflammation at 72 h but no overall difference in outcome
Ju *et al*[20], 2002	APAP (mouse)	Liposome/clodronate	Increased ALT at 8 and 24 h in treated group	Macrophage depletion increased liver damage
Holt *et al*[6], 2008	APAP (mouse)	CCR2 -/-	ALT same at 10 and 24 h; Comparable histological necrosis at 24 h but delayed recovery at 48 and 72 h in CCR2 -/-	Reduction in infiltrating macrophage population causes delayed recovery
Karlmark *et al*[5], 2009	CCl_4 (mouse)	Liposome/clodronate	Unaltered ALT level at 4 and 24 h post-CCl_4	Reduction in infiltrating macrophages had no effect on severity of liver damage

ALF: Acute liver failure; ALT: Alanine transaminase; WT: Wild-type; KO: Knockout; APAP: Acetaminophen induced hepatotoxicity; CCl_4: Carbon tetrachloride; CCR2: Chemokine (C-C motif) receptor 2.

flammatory effector cells including neutrophils, NK cells, NKT cells, and T cells that mediate tissue injury. However, there is some evidence to suggest that the population of hepatic macrophages derived from newly recruited infiltrating monocytes possess anti-inflammatory activity and may play a role in recovery from acute liver damage. Though it is beyond the scope of this review to discuss in detail, evidence also suggests that it is this same population of immigrant macrophages that is responsible for driving the fibrosis that follows acute liver injury[5,10].

THE BALANCE BETWEEN PRO- AND ANTI-INFLAMMATORY CYTOKINES

Macrophage-derived inflammatory cytokines in experimental models of ALF (Table 2)

Tumour necrosis factor-α: Tumour necrosis factor-α (TNF-α), a macrophage derived pro-inflammatory cytokine, has a central role in the pathogenesis of many inflammatory clinical syndromes. TNF-α can enhance the production of other pro-inflammatory cytokines and provoke cellular recruitment to sites of injury. It also has an important direct effector function by inducing hepatocellular apoptosis through its interaction with TNF-receptor 1. This cell surface receptor is connected to an intracellular death domain whose activation ultimately leads to the induction of effector caspases and the execution of cellular apoptosis.

In experimental models of acute liver failure, elevated serum levels of TNF-α and increased mRNA have been observed shortly after the initiating insult[21,25,48]. Studies in the murine Gal/LPS and Con-A models of hepatic failure have shown that treatment with neutralising anti-TNF antibodies provides protection against liver damage[24,25]. A recent study using targeted antisense oligonucleotides to inhibit expression of the TNF-α gene, limited the hepatic damage and subsequent mortality from Gal/LPS in a rat model of acute hepatic failure[26]. TNF-α-driven hepatocyte necrosis through the TNF-R1 pathway has been well described in a number of experimental models of acute liver injury[21,23,49].

Interferon-γ: Interferon-γ (IFN-γ) is a pro-inflammatory cytokine released by T-cells, NK cells, and macrophages. It has a variety of anti-viral, anti-bacterial and pro-inflammatory effects. In models of acute liver injury, increases in serum IFN-γ and intrahepatic IFN-γ mRNA are seen as part of the initial response to injury[28,29]. IFN-γ -/- knockout mice show resistance to toxic and T-cell mediated acute liver failure, with improved survival and reduced hepatic necrosis and apoptosis[30]. Neutralisation of IFN-γ with antibodies in WT mice confers a dose-dependent protective effect following APAP or Con-A administration[28,29].

IFN-γ deficient mice show a decrease in Fas induction after liver injury; hence it has been postulated that IFN-γ contributes to liver injury by the upregulation of Fas on hepatocytes, rendering them susceptible to apoptotic cell death[30].

Macrophage migration inhibitory factor: Macrophage migration inhibitory factor (MIF) is a multifunctional inflammatory cytokine that is constitutively expressed in many cell types, including centrilobular hepatocytes and Kupffer cells[50]. Pre-formed MIF is released early in response to stress (glucocorticoid) and infectious stimuli (LPS). It has an important role in stimulating the inflammatory response through cellular recruitment, stimulation of cytokine production, and counteracting the inhibitory effect of glucocorticoid on the production of pro-inflammatory cytokines. Though initially described as a T-cell cytokine, it has since been

Table 2 Pro- and anti-inflammatory cytokines in experimental models of ALF

Cytokine	Model	Source	Mechanism of action
TNF-α	Murine hepatic ischaemia[21,22] Murine α-amanitin or actinomycin D[23] Murine Gal/LPS[24] Murine Con-A[25] Rat Gal/LPS[26]	Macrophage	Hepatocellular apoptosis Cellular recruitment
IFN-γ	Murine Gal/LPS[27] Murine APAP[28] Murine Con-A[29,30]	Macrophages NK and T cells	Induce iNOS Upregulates Fas, sensitising hepatocytes to apoptosis Upregulates adhesion molecules and chemokines resulting in leucocyte accumulation
MIF	Guinea pig halothane[31] Murine APAP[31] Murine LPS in BCG-primed mice[32] Rat ethanol[33]	Preformed stores released from hepatocytes and KCs early in injury	Stimulate release of proinflammatory cytokines Counter-act glucocorticoid anti-inflammatory effects Cellular recruitment
IL-6	Murine APAP[22,34,35] Murine alcohol and TNF-α[36] Murine Con-A[37,38] Murine Acute CCl_4[39] Murine Fas-mediated apoptosis[40] Murine ischemia/reperfusion[41]	Released from macrophages and T cells	Reduced TNF-α secretion Activation of STAT3 signalling pathway Induction of anti-apoptotic proteins e.g. Bcl-2, Bcl-xL, FLIP
IL-10	Murine Gal/LPS[27,42,43] Murine CCl_4[44] Rat CCl_4[45] Murine APAP[46] Murine Con-A[47]	Macrophages/monocytes and injured hepatocytes	Inhibition of TNF-α, IFN-γ secretion

Con-A: Concanavalin-A; Gal/LPS: Galactosamine lipopolysaccharide; TNF-α: Tumour necrosis factor-α; IFN-γ: Interferon-γ; MIF: Macrophage migration inhibitory factor; IL-6: Interleukin-6.

established that macrophages are an important source of MIF[51]. An increase in MIF mRNA is seen in KCs following acute injury[50].

MIF has a role in a number of experimental models of acute liver injury[31-33]. Significant increases in serum MIF are detectable in the early stages following acute liver injury by toxins or LPS[31,32]. MIF -/- knockout mice demonstrate resistance to APAP-induced hepatotoxicity, displaying lower ALT levels, less histological necrosis, and improved survival compared to WT counterparts[31]. In a BCG-LPS model of murine ALF pre-treatment with anti-MIF antibodies prevented the development of liver failure and death in response to LPS challenge[32].

Interleukin-6: Interleukin-6 (IL-6) is a macrophage and T cell-derived multifunctional cytokine with both pro- and anti-inflammatory actions. In a number of acute liver injury models, IL-6 has been shown to play an important protective role. IL-6 -/- knockout mice display increased sensitivity to liver injury, impaired regeneration, and poor outcomes in APAP[22,34], alcohol and TNF-α-mediated liver cell apoptosis[36], acute CCl_4 toxicity[39], Fas-mediated apoptosis[40], and ischaemia-reperfusion injury[41]. In a number of these experiments, the treatment of knockout and wild-type mice with recombinant IL-6 prior to the liver insult conferred protection against hepatic damage[37,39-41]. Experimental evidence suggests the hepatoprotective effects of IL-6 in acute liver damage are mediated through the activation of the STAT3 signalling pathway, with induction of anti-apoptotic proteins such as Bcl-2, Bcl-xL, and FLIP and inhibition of NKT cells *via* targeting of $CD4^+$ T cells[38,39,52].

Interleukin-10: IL-10 is a pleiotropic anti-inflammatory cytokine that is capable of downregulating several aspects of the pro-inflammatory cascade, including activated macrophage function. IL-10 is secreted from various cell types, including monocytes and macrophages, in response to injury. The liver is a major source of IL-10 in systemic inflammatory conditions.

In experimental models of acute liver injury, IL-10 has consistently been shown to be protective against liver damage[27,42-47]. IL-10 serum levels and mRNA expression in liver tissue are seen to rise early after hepatic insult in APAP, Con-A, and Gal/LPS models[42,46,47]. The kinetics of IL-10 production parallel TNF-α secretion in a murine Gal/LPS model, suggesting both pro- and inflammatory cytokines are released together and act concurrently during the early acute inflammatory response[42].

In a Con-A model of ALF, neutralisation of IL-10 with monoclonal antibodies exacerbated liver damage, as measured by serum transaminases and liver histology. Serum levels of TNF-α, IFN-γ, and IL-12 were augmented by neutralisation of IL-10[47]. Similar findings were demonstrated in an APAP model utilising IL-10 knockout mice, where the absence of IL-10 was associated with increased ALT, worse histological necrosis, and reduced survival. Again this increased susceptibility seemed to be attributable to upregulation of pro-inflammatory cytokines and the effectors TNF-α, IL-1, and iNOS[46]. Treatment with recombinant IL-10 in Gal/LPS models caused a dose-dependent reduction in liver damage and hepatic and serum TNF-α expression[27,42,43]. *In vitro* work on isolated rat hepatocytes demonstrated that IL-10 was expressed early following LPS challenge. Treatment of

KCs with exogenous IL-10 downregulated production of superoxide dismutase and TNF-α responses following LPS challenge; a phenomenon analogous to the functional monocyte deactivation described later[45].

Thus, in animal models of acute liver injury there is consistent evidence that IL-10 is expressed in the early phases of liver damage, simultaneous to pro-inflammatory effectors. It acts to abrogate liver injury by limiting the release of pro-inflammatory cytokines and effectors. *In vivo* it is likely to be the balance of these inter-related and mutually regulating factors that determine the extent and outcome of the hepatic insult.

Macrophage-derived inflammatory cytokines in human studies of ALF

As in experimental models of acute liver failure, ALF in humans is known to be associated with massive activation of pro- and anti-inflammatory mediators. A number of studies have looked at patient populations and correlated serum levels of inflammatory cytokines with clinical parameters of ALF severity. TNF-α has been shown in a number of clinical cohorts to be elevated in patients with ALF compared with controls with acute hepatitis or chronic liver disease[53-57]. In two of these studies, elevated TNF-α levels correlated with poor clinical outcomes[53,58]. IL-6 and IL-10 have been shown to possess hepatoprotective properties in experimental models of ALF models[53-56,59]. However, human studies of ALF have detected higher levels of IL-6 and -10 in non-surviving ALF patients compared to spontaneous survivors[53,56,59].

Interestingly, a number of recent studies have measured proxy markers of macrophage and monocyte activation and shown that increases in these markers correlate with poor outcome in patients with ALF. CD163 is a macrophage lineage-specific scavenger receptor that is expressed by activated macrophages (and to a lesser extent by circulating monocytes) and can be shed in the circulation as a soluble receptor[60]. Hiraoka *et al*[60] showed that, in a small population of patients with ALF, mean levels of serum CD163 were elevated when compared with either healthy controls or patients with acute hepatitis. There was a trend towards increasing CD163 in non-survivors, however this did not reach significance. Møller *et al*[61] used a larger cohort from the US ALF study group and replicated the finding of elevated CD163 in ALF patients compared with controls. They also showed a significant difference between CD163 levels on day three between survivors and non-survivors.

FUNCTIONAL MONOCYTE DEACTIVATION IN HUMAN ALF

Infectious complications are a common feature of established acute liver failure and account for a significant proportion of overall mortality[1,62]. A number of recent studies have investigated the phenomenon of functional monocyte deactivation that occurs in patients with acute and acute-on-chronic liver failure, and that might be partially responsible for the observed susceptibility of these patients to overwhelming sepsis[53,63-65].

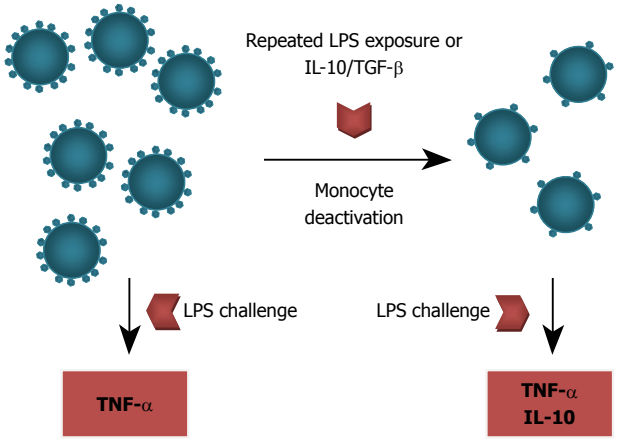

Figure 2 Circulating monocytes express abundant cell surface HLA-DR. Initial exposure to LPS provokes a massive tumour necrosis factor-α (TNF-α) response. Repeated exposure to LPS or the presence of interleukin-10 (IL-10)/transforming growth factor-β (TGF-β) causes monocyte deactivation with downregulation of cell surface HLA-DR expression, impaired antigen presenting capability, and an attenuated TNF-α response to future LPS challenge.

Monocyte deactivation was first described in the early 1990s in clinical populations of post-trauma and sepsis patients[58,66]. This phenomenon shares many features with the experimental model of "endotoxin tolerance", which is characterised by a refractory monocyte phenotype with decreased expression of HLA-DR, impaired ability to present antigens, and a profoundly attenuated TNF-α response to LPS challenge (Figure 2). This monocyte phenotype can be induced experimentally by repeated LPS challenge (hence endotoxin tolerance) or exposure to IL-10 and TGF-β[66].

Antoniades *et al*[53] demonstrated monocyte deactivation in a group of patients with acetaminophen-induced acute liver failure (AALF). Patients with AALF who had adverse outcomes (death or transplantation) had significantly lower expression of HLA-DR than transplant-free survivors with AALF, control patients with chronic liver disease, and healthy controls. A reduction in the percentage and total number of HLA-DR positive monocytes was observed, as was a reduction in HLA-DR expression on individual monocytes. Longitudinal follow up of non-surviving and surviving patients showed the former group had persisting evidence of monocyte deactivation, with suppressed HLA-DR at days 3-6, whereas the later group showed recovery of monocyte function between day 1 and days 3-6.

Three studies have looked at this phenomenon in patients with acute-on-chronic liver failure and shown a similar reduction in monocyte HLA-DR in this group[63-65]. Furthermore, they all demonstrated that monocytes derived from acute-on-chronic liver failure patients show impaired *ex vivo* TNF-α secretion in response to LPS exposure. Berres *et al*[64] and Xing *et al*[65] also replicated the finding that low monocyte HLA-DR has a strong association with poor outcome in liver failure patients.

The confirmation of immune dysfunction with functional monocyte deactivation in ALF begs the question

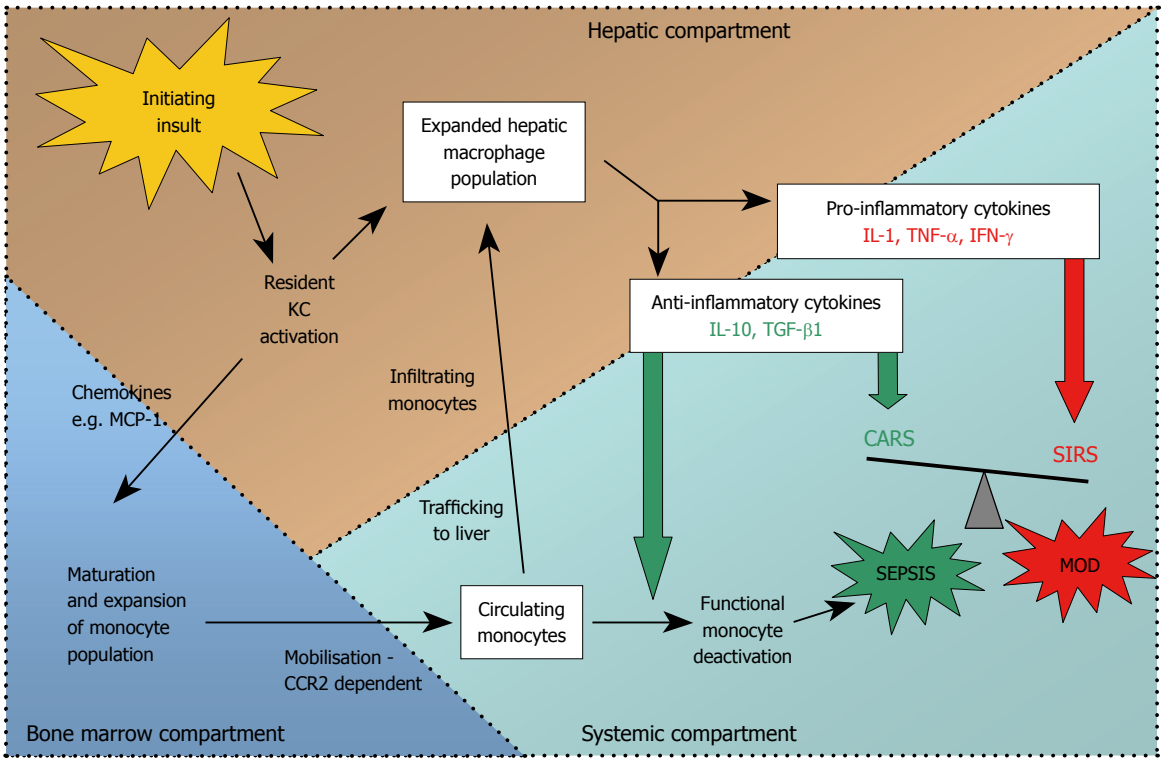

Figure 3 Summary of pathways. The initiating hepatic insult causes early release of chemokines, which promote the maturation of a population of bone marrow monocytes. These activated monocytes enter the circulation and some will traffic to the liver and differentiate into macrophages, accounting for the marked expansion in hepatic macrophage numbers seen after acute liver injury. Pro and anti-inflammatory cytokines are released simultaneously, early in the course of ALF. High levels of both pro and anti-inflammatory cytokines can be measured in the systemic circulation and drive the systemic inflammatory response (SIRS) and compensatory anti-inflammatory response (CARS) respectively. Imbalance in these two opposing forces in favour of an anti-inflammatory milieu can lead to functional monocyte deactivation, recurrent sepsis and multiple organ dysfunction (MOD). MCP-1: Monocyte chemotactic protein-1; CCR2: Chemokine receptor 2.

of what is driving this phenomenon and how might it be modulated to improve clinical outcomes. The presence of high levels of circulating anti-inflammatory cytokines, IL-10, TGF-β as part of the compensatory anti-inflammatory response (CARS) in ALF are obvious culprits, in that *in vitro* studies have clearly demonstrated their ability to induce monocyte deactivation (Figure 2). Thus it appears that the anti-inflammatory cytokines that provided early hepatic protection in acute liver failure, can, if unchecked, cause subsequent systemic immune paralysis that leads to a worse outcome.

CONCLUSION

Monocytes and macrophages are central players in the complex process of initiation, propagation, and resolution of acute liver injury. Current evidence from experimental and human models suggests that macrophages are activated early in the evolution of acute liver injury and respond with vigorous release of chemokines and pro-inflammatory cytokines that drive the acute inflammatory response to injury. Simultaneously, macrophages initiate a counter-acting anti-inflammatory cascade that moderates the degree of acute hepatic inflammation. As ALF progresses, circulating monocytes are recruited to the liver and are implicated in the resolution of inflammation and tissue repair processes. Meanwhile, the intricate balance between pro- and anti-inflammatory cytokines released from blood monocytes, hepatic macrophages, and other immune cells, is maintained through multiple interrelated regulatory mechanisms. However, in ALF, the immune response is skewed towards an anti-inflammatory-predominant environment, which could account for functional monocyte deactivation encountered and its attendant clinical sequelae of recurrent sepsis and multiple organ failure (Figure 3).

FUTURE DIRECTIONS

Further studies should aim to dissect the temporal relationship of macrophage recruitment and activation, in both circulating and hepatic inflammatory compartments, during the evolution of acute liver failure. Insight into the mechanisms that influence macrophage function during the process of acute hepatic injury will enable the development of therapeutic strategies that can be administered in a timely fashion to modulate the activity of this pivotal immune cell during acute liver failure.

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EDITORIAL

Modifier-concept of colorectal carcinogenesis: Lipidomics as a technical tool in pathway analysis

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Abstract

In the modifier concept of intestinal carcinogenesis, lipids have been established as important variables and one focus is given to long-chain fatty acids. Increased consumption of long-chain fatty acids is in discussion to modify the development of colorectal carcinoma in humans. Saturated long-chain fatty acids, in particular, are assumed to promote carcinogenesis, whereas polyunsaturated forms are likely to act in the opposite way. At present, the molecular mechanisms behind these effects are not well understood. Recently, it has been demonstrated by lipidomics and associated molecular techniques, that activation and metabolic channeling of long-chain fatty acids are important mechanisms to modify colorectal carcinogenesis. In this Editorial, an overview about the present concept of long-chain fatty acids and its derivatives in colorectal carcinogenesis as well as technical algorithms in lipid analysis is given.

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Key words: Colorectal neoplasms; Carcinogenesis; Long-chain fatty acids; Acyl-CoA; Lipidomics

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INTRODUCTION

Colorectal adenomas and carcinomas are much more common than small bowel tumors, and colorectal cancer (CRC) is a leading cause of cancer deaths in Europe and the United States. The clinical symptoms of bleeding and/or obstruction found in the presence of large intestinal neoplasias are similar regardless of histologic type or etiology. It has been suggested that there is an association between colorectal carcinogenesis and pathways of fatty acid metabolism, with long-chain acyl-CoA thioesters as essential intermediates and cyclooxygenases (COXs) and acyl-CoA synthetases (ACSLs) as important enzymes. This observation is determined by molecular data indicating high functional diversity of activated lipid intermediates including formation of complex lipids (e.g. ceramide) and utilization in beta-oxidation as well as regulation of cellular signaling, transcriptional activity, cellular structure, and cellular behavior. Dietary lipids have been assumed as important additional variables in colorectal carcinogenesis.

In the following paragraphs the modifying role of lipids in colorectal carcinogenesis and the innovative technical approaches to elucidate the complex molecular network of lipids are addressed in detail.

EPIDEMIOLOGY AND DIVERSITY OF COLORECTAL CANCER

CRC continues to be one of the most common malignancies worldwide and the third leading cause of death among cancers[1]. In industrialized Western countries, the incidence and prevalence are higher than in Asia or developing countries. Although the molecular mechanism of CRC has become better understood in the past 2 decades, the prognosis of CRC, especially advanced cancer, has not significantly improved. Five-year survival rates have risen from 56.5% for patients diagnosed in the early 1980s to only 63.2% for those diagnosed in the early 1990s, and most recently to 64.9%[2]. The sparsely improving trend is most likely due to the fact that the prognosis for patients with CRC is highly dependent on disease stage at the point of diagnosis. Only 10% of CRCs are diagnosed early, and in most patients advanced disease is found. Therefore, early detection of CRC is essential in improvement of a patient's prognosis.

CRCs comprise a heterogeneous group of malignancies including hereditary forms and so-called sporadic tumors[3,4]. At the current state of knowledge, the majority of CRCs are sporadic. However, experimental and epidemiological data give evidence that the class of sporadic CRCs comprises a heterogeneous group of malignancies with differences in pathogenesis and prognosis. It is suggested that the pathogenesis of some sporadic CRCs does not follow the traditional adenoma-carcinoma sequence. Recently, a new pathway in "sporadic" CRC development, the serrated pathway, has been discovered[5]. The molecular alterations seen in serrated tumors are different from those traditionally found in the adenoma-carcinoma sequence. In serrated lesions, loss of heterozygosity and mutations in *APC* or *TP53* are rare, whereas hypermethylation of CpG islands and microsatellite instability are frequent. At present, 2 categories of serrated carcinogenesis are defined including 2 basic pathways, the sessile serrated pathway and the traditional serrated pathway. While in the sessile serrated pathway *BRAF* mutation and high-level microsatellite instability are molecular hallmarks in carcinogenesis, and tumor location is predominantly in the right colon, *KRAS* mutation and microsatellite stability have been characterized as important steps in the traditional serrated pathway, where cancers arise mainly in the left colon.

The diversity in colorectal carcinogenesis is accompanied by numerous genetic and epigenetic alterations. Epidemiological data indicate that the heterogeneity of such genomic lesions is determined by additive factors, called the modifiers of colorectal carcinogenesis[6]. Evidence is given that diet and nutrition are key factors acting as carcinogenesis modifiers. This activity is probably relevant in both steps of colorectal tumorigenesis, initiation and progression. Therefore, identification and characterization of nutritional components as promoters or silencers of intestinal cancer are of high interest in tumor preventive strategies, tumor epidemiology, and the health economy.

THE MODIFIER CONCEPT OF COLORECTAL CARCINOGENESIS

At present, the model of tumor genotype to phenotype/histomorphology correlation, best-known as the adenoma-carcinoma sequence, is well accepted. The genetic changes that occur in colorectal carcinogenesis are paralleled by the progression from small premalignant lesions to advanced carcinomas indicating that each successive genetic injury/modification confers an advantageous characteristic upon the expanding tumor. The MAPK-ERK (mitogen-activated protein kinase-extracellular signal-regulated kinase) pathway, which mediates cellular responses to many extracellular inputs, is involved in regulation of apoptosis, cell growth, secretion, and cellular differentiation. K-ras and B-raf are key players in this signaling cascade, because mutations result in continuous gene activation leading to autonomous cell proliferation and tumor growth. The *KRAS* gene belongs to the Ras oncogene family and encodes a 21 kDa protein with intrinsic GTPase activity. This protein is regulated by a cycle of de- and re-palmitoylation, which promotes its rapid exchange between the plasma membrane and the Golgi apparatus. While numerous different genes can become altered in carcinogenesis, it has been recently proposed that the number of deregulated signaling pathways is relatively small[7].

Over the last few decades it has been shown that aberrant activity of some cellular pathways is crucial in colorectal carcinogenesis. Alterations in the coding genes, e.g. *APC* or *TP53* mutations and loss of heterozygosity, were elucidated as key mechanisms for misdirected signaling[8]. Important insights into such principles of cancer-relevant molecular signaling were given by the characterization of hereditary colorectal carcinoma syndromes, i.e. hereditary non-polyposis colon cancer, familial adenomatous polyposis, and MUTYH-associated polyposis. Genotype to phenotype correlations in affected patients revealed that the type of variation in gene sequence and epigenetics is essential for onset and progress in cancer development. However, some inter-individual discrepancies were found in this correlation analysis, indicating additional molecular mechanisms that are likely to modify pathway execution[9-11]. These observations were substantiated by several experiments including studies in *APC* mice revealing examples of modifying genetic loci and non-genetic co-factors, e.g. nutritional components[12]. In mice, modifying genetic loci were addressed with the acronym Mom (modifiers of min) and demonstrated the genetic-based principle of modulation in gene expression with relevance to disease severity. One interesting candidate gene, firstly identified in the *APC* mice, is *Pla2g2a* coding secretory phospholipase A2 (PLA2)[13]. Activity of this enzyme (prototype group Ⅰ) is associated with diverse functions including digestion, smooth muscle contraction, and cell proliferation. In addition, PLA2s of prototype group Ⅱ are involved in inflammatory conditions and are upregulated

by pro-inflammatory cytokines such as tumor necrosis factor and interleukin-1B. Recently, the PLA2-receptor (PLA2R) has been identified as a potential new tumor suppressor gene crucial in the induction of cellular senescence through activation of the p53 pathway[14].

In addition to genes acting as defined modifiers in colorectal carcinogenesis, matrix composition has been identified as an important factor to modify epithelial cell behavior and intestinal tumorigenesis[15]. The heterogeneity in matrix composition is marked and is a result of the complex mixture of non-epithelial cells embedded in a well-adapted set of extracellular stored lamellar and non-lamellar molecules. Additionally, different types of secreted intestinal products, such as mucins and other glycoproteins which are non-cellular matrix-components have been identified as important modifiers in intestinal pathogenesis[16]. Muc2, expressed by goblet cells, is the most abundant secreted intestinal mucin and important in intestinal homeostasis. The impaired expression and/or secretion of Muc2 have been correlated with the development of inflammatory intestinal conditions and colorectal carcinogenesis[17-20]. Recently, the molecular basis for this phenomenon was identified, when an interaction of Muc2 and Apc resulting in modification of Wnt signalling was determined[21,22].

In addition to Muc2, other molecules such as cytokines are suggested as important mediators in chronic intestinal inflammation and modifiers of intestinal tumor development. In the intestine, the main sources of cytokines and inflammation-triggered molecules are lymphocytes. The pathophysiological association of chronic inflammatory activity and intestinal tumorigenesis has been substantiated by epidemiological data demonstrating a high incidence of CRC in patients with chronic inflammatory bowel disease, a condition additionally associated with disturbances in composition of non-cellular matrix components.

Over the last decade, an additional modifier of colorectal carcinogenesis and pathogenesis of non-neoplastic intestinal diseases has been identified and characterized, namely the microbiome. The term intestinal microbiome describes the taxonomically complex and micro-ecological highly dynamic community of microorganisms on the gut mucosal surface[23]. The interaction of surface lining mucosal cells and the microbiome has an impact on the metabolome and lipidome, the maturation and proliferation of intestinal cells along the crypt-plateau axis, tissue homeostasis, and can be a factor in various diseases, such as inflammatory bowel disease and obesity[24,25]. The successful use of designed bacterial strains in the treatment of inflammatory intestinal conditions, such as necrotizing enterocolitis in preterm infants, underline that the microbiome modificatory activity is fundamental in intestinal homeostasis[26].

In summary, colorectal carcinogenesis is based on a sequence of molecular events in enterocytes including gene mutations, epigenetic modifications, and aberrant signaling in basic cellular pathways. However, proliferation, cellular activities, and cell death of enterocytes and the execution of so-called modifier genes of CRC-relevant pathways strongly depend on additional variables including matrix composition, the microbiome, chronic inflammatory activity, environmental effects, and dietary components. In particular, nutrition and diet, as stated below, are suggested as fundamental in modifying the initiation and progression of CRCs[6]. Key aspects in the current view of the CRC carcinogenesis modifier concept are summarized in Figure 1.

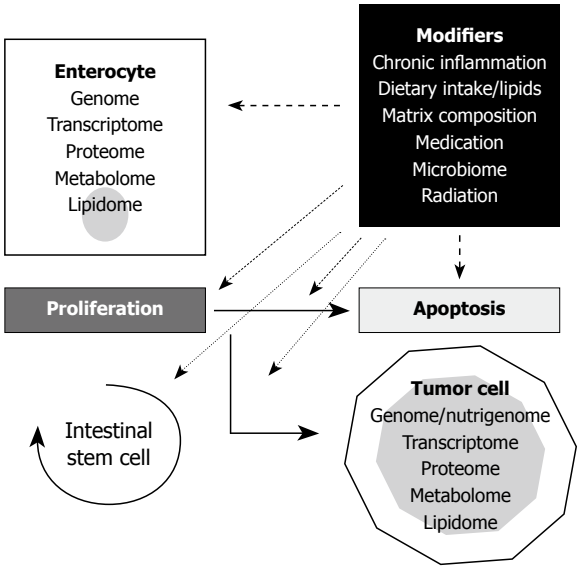

Figure 1 The modifier concept of colorectal carcinogenesis. The important key points in enterocytic life span, proliferation and apoptosis, are connected by basal molecular pathways. Such pathways, as well as cell behavior determining molecular entities such as the genome, transcriptome, proteome, metabolome, and lipidome, are exposed to cellular stress and act as modifiers. In addition to enterocytes, the intestinal stem cells are affected by this evolutionary principle. The aberrant modification of pathways is suggested as an important variable in colorectal carcinogenesis. The term nutrigenome of tumor cells reflects the genome modification of malignant cells by dietary components with expression of so-called modifier genes as demonstrated in *APC* mice.

NUTRITION AND CRC - DIETARY FAT AND INTESTINAL LIPIDS

Among the many aspects of industrialization, such as obesity, exposure to electromagnetic fields, radiation, medications, and a reduced physical activity, diet seems to be a plausible variable in colorectal carcinogenesis because of its direct contact with the intestinal mucosa, i.e. the surface lining epithelial cells[27]. The literature is marked by epidemiological studies aiming to elucidate the constituents of a CRC-preventive diet, the optimal amount of dietary fiber, and to define biologic markers for nutritional epidemiology of CRC[28]. A reason for the controversial outcome of such studies could be that the entire diet, with its balance of animal and vegetable products, and grains that have been processed to varying degrees, is highly variable and not a single entity. However, some dietary components are described as putative candidates in modifying colorectal carcinogenesis. An increased risk for cancer development is found in individuals consuming diets high in red and processed meat[29]. In

addition, alcohol in combination with a diet low in folate and methionine appears to increase cancer risk[30-32]. Vitamin D and calcium are suggested to have a protective effect in CRC carcinogenesis, but the molecular mechanism behind this has not been elucidated. Recently, it has been shown that deep-fried/oxidized fats such as hydroxyl- and hydroperoxy fatty acids, are able to influence lipid metabolism by activation of the transcription factor, peroxisome proliferator-activated receptor α (PPARα)[33]. Oxidized fats are established in diets containing large quantities of polyunsaturated fatty acids (PUFAs) by heating. Expression of PPARα-related genes is involved in degradation of fatty acids, affects the synthesis of cholesterol/triacylglyceride, and is associated with cellular behavior. These data indicate that lipid metabolism activity and cellular lipid-dependent signaling could be modified by a diet enriched in deep-fried fats.

In the last few decades, the highly diverse class of lipids has become the focus of intensive research activities, because epidemiological data have indicated a link between the intake of dietary lipids and development of (sporadic) CRCs[34,35]. In this concept, the composition of the different types of fatty acids and the expression/activity of lipid-metabolizing enzymes such as COXs and ACSLs are of high importance[36]. A growing number of reports support the findings that bioactive dietary components containing long-chain PUFAs modulate important determinants that link inflammation to cancer development and tumor progression[37], whereas short-chain fatty acids, e.g. acetate, propionate, and especially butyrate, mainly produced by the microbiome using fermentable dietary polysaccharides, are suggested to be cancer-preventive[38,39]. Propionate and acetate are utilized preferentially by extra-intestinal tissues, whereas butyrate is suggested to play an important role in intestinal mucosa homeostasis as an energy source for enterocytes. It has been shown that cancer-preventive intestinal butyrate levels correlate with increased epithelial cell differentiation, cell cycle arrest, and apoptosis of enterocytes. Additional aspects of butyrate activity, namely inhibition of the enzyme histone deacetylase, and a decrease in the transformation of primary to secondary bile acids as a result of colonic acidification, could be of relevance in colorectal carcinogenesis[40]. Recently, multiple free fatty acid receptors (FFAR) have been identified in the intestine[41]. The receptor activity highly depends on the length of the free fatty acids. Short-chain fatty acid signaling is mediated by FFAR2 and FFAR3, whereas FFAR1 and GPR120 can be activated by medium- and long-chain free fatty acids. It is suggested that defects in FFAR3 are associated with reduced extraction of energy from short-chain free fatty acids. The first experimental evidence was that at least FFAR2 could be associated with cellular transformation and colorectal carcinogenesis. In this experimental setting the putative mitogenic action of FFAR2 was aggravated by short-chain free fatty acids.

The important role of lipids, especially fatty acids, in the modifier concept of CRCs is underlined by the plethora of intestinal long-chain fatty acid (LCFA) activities. LCFAs were suggested as important cell cycle modifiers, particularly in enterocytes. In ACSL5-overexpressing enterocytes, an increase in apoptosis susceptibility was found, probably as a result of an aberrant and locally restricted increase in LCFA derivatives[42]. These investigations underline the importance of ACSL-mediated metabolic channeling of fatty acids in the regulation of cell behavior[43]. In addition, lipidation of proteins is suggested as a basic mechanism in the translation of LCFA modifier activities in several signaling cascades and receptor structures[44]. This mechanism was recently demonstrated for the CD95 death-inducing signaling complex (DISC)[45]. In the earliest events in DISC formation, palmitoylation of CD95 on cysteine 199 enhances receptor aggregation to a high molecular complex. Palmitoylation, as an important mechanism in fatty acid-based protein modification *via* thio-ester linkage to cysteine (S-palmitoylation), has been recently classified into 5 general classes[46]. The first class comprises transmembrane proteins S-acylated on cysteines adjacent to or in the transmembrane domain. In the second class, proteins are typified by Ras family molecules, where S-palmitoylation is dependent on prior prenylation within the C-terminal "CAAX" box. In the third class, palmitoylated proteins include molecules with S-palmitoylation at one or more cysteine residues near the N- or C-terminus. Dual acylation is found in the fourth class proteins such as members of the Src and G protein families. N-myristoylation is suggested to improve accessibility of the protein to a membrane-bound palmitoyl acyl transferase and subsequent protein palmitoylation. The hallmark of class 5 proteins is covalent binding of palmitate *via* amide-linkage to an N-terminal cysteine residue.

In summary, dietary fatty acids are suggested as important substrates for protein modification *via* direct molecular interaction. A complex metabolic conversion of absorbed lipids into cell-synthesized LCFA is not mandatory. However, synthesis of acyl-CoA derivatives from dietary fatty acids seems an important step for the modifying activity and bioavailability of lipids. Palmitoylation has been shown as a basic molecular mechanism in lipid-dependent protein modification. As detailed below, lipidomics is a powerful technical algorithm to elucidate key aspects in the interaction of dietary lipids and cellular lipid metabolism.

LIPIDOMICS - A PROMISING TOOL IN CRC-RELATED LIPID ANALYSIS

In the last 2 decades, laboratory technology to investigate carcinogenesis has primarily focused on genomic and proteomic analyses[47]. Many research-based studies on CRC combined genetic, translation-related and/or biochemical approaches to discover clinically useful DNA- or protein-based biomarkers for CRC screening, diagnosis and response to treatment. Since the crucial roles of lipids in numerous signaling pathways including carcinogenesis, apoptosis and proliferation, has been identified, the necessity to understand and analyze the impact of lipids in

colorectal carcinogenesis, diagnosis, prognosis and treatment becomes more and more important.

In recent years, lipidomics, a novelty in the "-omic" research, has emerged as a promising tool to investigate cellular lipid metabolism and pathogenic alterations in lipid pathways[48]. Lipidomics, defined as the large-scale study of the pathways and networks of cellular lipids, is a chromatography- and mass-spectrometry-based research area that includes the study of cellular and functional lipidomics as well as lipids in health and disease. The cellular lipidome comprises over 1000 different, highly diverse and complex lipids of which many are metabolically interconvertible and structurally similar[49]. Because of this complexity, comprehensive lipid analyses are required to elucidate the physiological properties and functionality of different lipid metabolites. The ability to analytically distinguish different lipid species is critical. Despite these experimental challenges and the fact that lipidomics has only emerged as a distinct field within the past few years, the number of compounds that can be analyzed is growing rapidly[50]; including fatty acids, sphingolipids, glycerolipids, glycerophospholipids, sterol lipids and prenol lipids, each containing distinct classes and subclasses of molecules[51-56]. The following section gives a short overview of classical and state-of-the-art analytical techniques that are commonly used by lipidologists to investigate lipids in health and disease.

Thin layer chromatography (TLC) has been used for the qualitative study of lipids since the early 1960s[57,58]. The technique uses a thin layer of a stationary phase such as silica or cellulose on a flat support, usually a glass or aluminum plate. Although the combination of a wide variety of silica-based solid-phase and organic mobile-phase protocols enables the separation of almost all lipid classes, TLC does not offer sufficient structural specificity and sensitivity as other methods of lipid detection. This has led to its use primarily as a screening tool prior to use of more sensitive and elaborated methods.

High pressure liquid chromatography (HPLC), a more sophisticated chromatographic technique, is routinely employed in lipidomic analysis for separation and identification of lipid species. HPLC provides resolution of lipids based on headgroup/class (normal phase) or fatty acid residues (reverse phase). For example, fatty acids and acyl-CoA are almost always separated on a reverse-phase column where the separation occurs on the basis of polarity, effective chain length, and degree of unsaturation[51], whereas separation of phospholipids can be achieved by either normal-phase HPLC[59] or reverse-phase HPLC[60]. However, HPLC alone can not guarantee the accurate quantification of lipids since this often depends on definite resolution and structural elucidation.

Mass spectrometry based techniques are currently the analytical tool of choice in lipidomic methodology. Two powerful approaches that provide sensitivity and structural specificity are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. ESI allows lipids in solution to be continuously infused directly into the ion source of a mass spectrometer, typically a triple quadrupole mass analyzer. By applying a high voltage to the infusion capillary, an electric field is created along which the charged droplets travel. As they travel, the solvent rapidly evaporates and droplets divide into individual charged ions to enter the mass spectrometer. The combination of classical separation techniques such as HPLC with state of the art ESI-mass spectrometry provides the necessary quantitative accuracy, sensitivity and structural specificity to detect and quantify a wide range of lipid species. Thus, liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS)-based methods are currently one of the most popular technologies in lipid research[52,61-63].

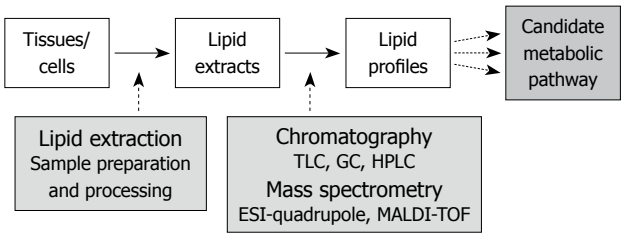

Figure 2 Summary of the lipidomics work flow - lipid extraction and analysis. TLC: Thin layer chromatography; GC: Gas chromatography; HPLC: High pressure liquid chromatography; ESI: Electrospray ionization; MALDI-TOF: Matrix-assisted laser desorption ionization-time-of flight.

In contrast to the LC-based lipid analysis, the recently developed direct infusion-based shotgun lipidomics approach, an analytical platform without direct coupling of any chromatography for lipid separation, allows the absolute quantification of hundreds of lipid species in small quantities with high throughput. This powerful strategy provides a promising future application in clinical studies and medical approaches to investigate pathogenic changes in lipid metabolism and lipid-mediated disorders[64,65].

In addition to ESI mass spectrometry, MALDI mass spectrometry, typically used in conjunction with time-of flight mass analyzers, is a laser-based soft ionization method that has been successfully used for large lipid species. Here, the lipid sample is mixed with an energy absorbing matrix and irritated with a laser to ionize the lipid molecules which then enter the mass spectrometer. Although MALDI is most often used for protein analyses and only used to a small extent for lipid analyses, the power of this technology lies in its application for tissue-imaging mass spectrometry. Direct tissue analysis of lipid species provides the opportunity to identify not only quantities but also the distribution and subcellular localization of lipids within the tissue[66,67]. MALDI tissue imaging (MSI), thus, greatly enhances the application spectrum of lipidomic analysis in disease-related alterations in lipid metabolism. A typical lipidomic work flow, including sample preparation and analysis is summarized in Figure 2.

The potential and the feasibility of lipidomic technologies in studies of diabetes, obesity, atherosclerosis and Alzheimer's disease have been described recently[68]. A lipidomic study of breast cancer pathogenesis demon-

strated that the complete profile of adipose tissue lipids in patients with benign and malignant breast tumors, rather than a single lipid has the capability to quantify the dietary part of breast cancer risk and to identify dietary modification in order to reduce breast cancer occurrence[69]. As the relevance of lipids as potential biomarkers becomes apparent, an increasing number of studies have focused on the discovery of reliable lipid biomarkers for disease prognosis, prevention and therapeutic application. A recent study showed that the analysis of fatty acyl chains from ethanolamine-containing plasmalogens provided a suitable biomarker to identify malignancy and metastatic capacity of frequent human cancers, such as breast, lung, and prostate cancer[70]. Although there are currently not many studies focusing on lipidomics-based approaches in colorectal carcinogenesis, the implications of this strategy for the future of CRC treatment and prevention are vast. As the field of lipidomics advances, the role of the lipidome in cellular functions and pathologic states will become clearer, and the identification and establishment of preventive and therapeutic approaches will become more focused. The integration of lipidomic data with genetic, proteomic and metabolomic data will provide a powerful analytical platform for elucidating the mechanism of lipid-based disease, for biomarker screening, and for monitoring pharmacological therapy[71].

CONCLUSION

In current opinion, the basic molecular pathways determining colorectal carcinogenesis are few in number. However, the number of mechanisms that could act in modifying initiation and progression of intestinal neoplasia is constantly growing. Among dietary components, the modifying capacity of lipids, especially of LCFAs, has been demonstrated by experimental and epidemiological studies. Lipidomics, the large-scale study of the pathways and networks of lipids in cells or tissues, is established as a modern and powerful tool to further elucidate the colorectal carcinogenesis modifying activities of lipids.

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OBSERVATION

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Risk factors in familial forms of celiac disease

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Abstract

Celiac disease has been reported in up to 2% of some European populations. A similar risk has been identified in the America and Australia where immigration of Europeans has occurred. Moreover, an increasing number of celiac disease patients are being identified in many Asian countries, including China and India. Finally, celiac disease has also been detected in Asian immigrants and their descendants to other countries, such as Canada. Within these so-called "general" celiac populations, however, there are specific high risk groups that have an even higher prevalence of celiac disease. Indeed, the single most important risk factor for celiac disease is having a first-degree relative with already-defined celiac disease, particularly a sibling. A rate up to 20% or more has been noted. Risk is even greater if a specific family has 2 siblings affected, particularly if a male carries the human leukocyte antigen-DQ2. Both structural changes in the small bowel architecture occur along with functional changes in permeability, even in asymptomatic first-degree relatives. Even if celiac disease is not evident, the risk of other autoimmune disorders seems significantly increased in first-degree relatives as well as intestinal lymphoma. Identification of celiac disease is important since recent long-term studies have shown that the mortality of celiac disease is increased, if it is unrecognized and untreated.

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INTRODUCTION

Only a few decades ago, celiac disease was considered an uncommon disorder present mainly in Europe or in countries where Europeans had emigrated, including Canada and Australia[1]. In Ireland, and in particular, Galway in the West of Ireland, appears to have an especially high prevalence of celiac disease with an estimate of up to 1 in 300 persons[2]. Similar experiences have been noted in Scandinavian countries. In the United States, however, earlier reports suggest that the detection of celiac disease is surprisingly low[1,3]. With the emergence of modern serological screening tests for celiac disease and increased efforts to use these screening measures, celiac disease has become more readily detectable.

EMERGING RISK ESTIMATES

In recent years, changes have occurred with an estimate of overall prevalence rates increased in most countries, often in the region of about 1%-2%[4]. Interestingly, in a longitudinally-based study from Olmstead County in the United States over many decades, increased detection has been reported, in part, due to increased use of serological screening[5]. Recently, a report from Hangzhou in China has also suggested that the prevalence of adult celiac disease may be more common in China than previously appreciated[6]. Of note, celiac disease has also been

reported in immigrants to Canada from China, Japan and South Asia, particularly from the Punjab region of India[7]. Even in the United States, celiac disease is now generally believed to affect 0.5%-1.0% of the general population[8].

HIGH RISK GROUPS FOR CELIAC DISEASE

These reported numbers often reflect prevalence estimates, usually resulting from screening of selected groups, e.g. blood bank serum samples, thought to be representative of the entire population from a specific geographic area. Confirmatory small intestinal biopsies have often been done in sero-positive cases to provide an overall "minimal" estimate of population prevalence (since all sero-positive patients may not be available for biopsy). As shown in Table 1, there are also some high risk groups within the general celiac population, often without typical clinical symptoms, such as diarrhea or weight loss, that may have even higher prevalence rates. Among these factors that specifically denote a higher risk for celiac disease, the single most important is a familial history of biopsy-defined celiac disease with some estimates up to 20% or more of first-degree relatives. Diagnosis of celiac disease in all of these high risk groups, however, is especially important since failure to detect celiac disease coupled with failure to treat the disease may lead to an increased morbidity, and critically, a nearly 4-fold increased risk of mortality from celiac disease[9].

Table 1 High risk populations for celiac disease

Relatives, especially first-degree
Anemia, especially iron deficiency
Osteopenic bone disease
Insulin-dependent diabetes (type 1), especially children
Liver disorders, especially AIH and PBC
Genetic disorders, including Down and Turner's syndrome
Autoimmune endocrinopathy, especially thyroid disease
Skin disorders, particularly dermatitis herpetiformis
Neurological disorders, including ataxia, seizures, MG
Others, including IgA nephropathy

AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; MG: Myasthenia gravis.

Table 2 Evidence of familial risk

Biopsy and serological studies
Irish Setter dog model studies
Functional permeability studies
HLA marker studies
Genome wide expression/linkage studies

HLA: Human leukocyte antigen.

FAMILIAL RISK FACTORS

Evidence for a familial risk in celiac disease has been accumulated from many sources (Table 2), including biopsy and serological studies in families with known celiac disease[10-17], animal model studies in the Irish Setter dog[18,19], functional studies based on evaluation of intestinal permeability or other absorptive indices[20-23], human leukocyte antigen (HLA)-genotyping studies[24-26], and more recently, genome-wide expression and linkage studies[27].

Biopsy and serological studies

Early family studies using small intestinal biopsies have provided strong evidence for the familial nature of celiac disease[10,11]. A systematic review of these earlier studies using MEDLINE and EMBASE databases concluded that up to 20% of first-degree relatives of European descent may be at risk for celiac disease[12]. Similar results have been reported from non-European populations, including a recent report from the Punjab region of India[13]. In addition, more extensive studies in biopsy-defined celiac disease have further confirmed these findings, particularly in "at-risk" first-degree (1:22) and second-degree relatives (1:39), compared to a control "not-at-risk" group (1:133)[14]. This risk appears to be especially increased in families with at least 2 siblings diagnosed as celiac disease[15], and in this setting, more males were detected compared to females[16]. Particularly significant predictors of familial risk include carrying HLA-DQ2 and being a sibling of the proband[17].

Animal model studies

Animal studies have provided some indirect, but important evidence. Gluten-sensitive enteropathy has been reported in a family of Irish Setter dogs[18] along with the association of a canine major histocompatibility complex (MHC) DQ haplotype[19].

Functional studies

Earlier studies showed that an intentional increase in gluten intake may provoke jejunal mucosal architectural changes in some first-degree relatives along with reduced xylose absorption, even in those without clinically overt disease[20], suggesting that, in the genetically-predisposed, both structural and functional changes may be induced in familial celiac disease. Abnormal permeability to lactulose and mannitol in first-degree relatives also suggests that functional changes may occur in biopsy-defined disease[21] as well as in first-degree relatives with apparently normal small bowel biopsies[22]. Importantly, this familial risk in first-degree relatives is genetically-based and not related to their daily calculated gluten intake[23]. In other words, familial celiac disease is not due to a common environment with increased intake of gluten-containing foods, but has a definitive genetic basis.

Human leukocyte antigen marker studies

HLA markers have been popularly used for decades in clinical studies of celiac disease. These clinical studies have served to emphasize the strong genetic component involved in the development of celiac disease. In large part, possession of the HLA-DQ2 variant, necessary for presentation of dietary antigens to intestinal T-cells, is

critical. Non-HLA genetic factors are also increasingly recognized as a very important, and possibly even a more significant and critical risk factor in the development of familial celiac disease. Furthermore, a recent HLA-genotyping study (i.e. DQA1*0501, DQB1*0201, DRB1*04) with small bowel biopsies from Finland has suggested a practical means of exclusion of some first-degree relatives without celiac disease for screening purposes[24]. However, it has been reported that some asymptomatic first-degree relatives may show only minimal changes in intra-epithelial lymphocyte numbers[25]. In addition, with a prolonged follow-up, flat small bowel mucosal biopsies have been reported in familial disease without initially abnormal biopsies[26]. These findings are important and need to be confirmed as further longitudinal studies may be required in first-degree relatives without initial biopsy evidence of celiac disease.

Genome-wide studies

Most exciting are the recent genome-wide expression and genome-wide linkage studies done to explore a number of clinical disorders, including celiac disease. A recent meta-analysis of 8 genome-wide linkage studies in celiac disease permitted definition of both HLA and non-HLA chromosome regions that appear to predispose to celiac disease and suggested the possibility of different types of disease-predisposing variants[27].

RISK OF ASSOCIATED DISEASE IN RELATIVES

Of potentially even greater significance are reports on relatives without celiac disease at risk for other diseases, often associated with celiac disease. For example, a serological survey study of first-degree relatives of children with documented celiac disease suggested that autoimmune diseases may be increased in addition to biopsy-defined, but "sub-clinical" celiac disease[28]. In addition, first-degree relatives may also develop the closely linked dermatological disorder, dermatitis herpetiformis[29]. Moreover, patients with type-1 diabetes have an increased risk of celiac disease and biopsy-defined, but essentially asymptomatic and unrecognized celiac disease[30]. Finally, although lymphoma risk seems to have fallen in the past 4 decades, individuals with a sibling affected with celiac disease have an increased lymphoma risk[31]. These studies, then, have important implications for risk of familial forms of celiac disease in relatives, particularly first-degree, but also for a host of other genetically-related clinical disorders, even in the absence of celiac disease. In future, expression of different celiac disease phenotypes and their individual specific risks of different diseases may be more readily defined with precise genetic markers or more precise genetic signatures.

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REVIEW

Chemokines and hepatocellular carcinoma

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Abstract

Chemokines play a paramount role in tumor progression. In hepatocellular carcinoma (HCC) progression, chemokines and their receptors play an intricate role. Currently, chemokines and their receptors such as the CXCL12-CXCR4 axis, CX3CL1-CX3CR1 axis and the CCL20-CCR6 axis have received much research attention. Although a large number of studies show that these axes are strongly associated with HCC, the exact mechanism by which these axes promote the growth and progression of HCC remains unknown. In this paper, several chemokines and their receptor interactions in HCC progression, growth and metastasis and immune response to HCC are reviewed.

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Key words: Chemokines; Hepatocellular carcinoma

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INTRODUCTION

Chemokines in humans are a family of small proteins of 45-50 kb which contain a structural homologous conservative family of cysteine residues. Chemokines are classified into four families, namely CXC, CC, CX3C and C according to the presence of four cysteine residues in conserved locations. CXC are subdivided into two categories, those with a specific amino acid sequence (or motif) of glutamic acid-leucine-arginine (or ELR for short) immediately before the first cysteine of the CXC motif (ELR-positive), and those without an ELR motif (ELR-negative). CXC and its cognate receptor are combined and activated. The receptors are mainly found in neutrophils, lymphocytes, endothelial cells and epithelial cells. Typical chemokine proteins begin with a signal peptide of approximately 20 amino acids that is cleaved from the mature portion of the molecule during its secretion from the cell. All chemokines share a typical 3-dimensional structure which is stabilized by two disulfide bonds joining the first to the third, and the second to the fourth cysteine residues. CC receptors are mainly distributed in dendritic cells, lymphocytes, macrophages, eosinophilic leukocytes, and natural killer cells, but they do not activate neutrophils. Currently, more than 50 kinds of chemokines have been found with only 20 kinds of chemokine receptors, therefore, in this system a considerable number of useless receptors (receptor, R)/ligand (ligand, L) interact with each other.

Chemokines play a central role in many biological events, such as embryonic development, wound healing, angiogenesis, Th1/Th2 development, leukocyte homeostasis, lymphatic organ development, inflammatory diseases, and tumors. Previous studies suggest that

chemokines are soluble molecules, and are involved in inflammatory response by regulating the transfer of white blood cells. Chemokines are now considered to play a critical and complicated role in a variety of cells such as tumor cells. Chronic inflammation is a long-term and low-grade injury caused by chemical factors, bacteria, and viruses, and there is a strong association between chronic inflammatory conditions in a particular organ and cancer specific to that organ such as colon cancer and ulcerative colitis, colorectal cancer and inflammatory bowel disease, pancreatic cancer and chronic pancreatitis, esophageal adenocarcinoma and Barrett's esophagus, liver cancer and hepatitis[1-3]. The most striking evidence of the direct regulation of tumors and tumor growth by chemokines is the over-expression of GRO (CXCL1, growth-related oncogene), α, β, γ in human melanoma cells, and its tumorigenicity in nude mice from *in vitro* research[4,5]. Recent studies have been focused on the roles of chemokines and their corresponding receptors in the growth and progression of tumors. The chemokines and their receptors involved in these processes are known as axes. At present, a large number of studies have demonstrated that the CXCL12-CXCR4 axis may be related to distant organ metastasis of tumors while the CCL21-CCR7 axis may be associated with lymph node metastasis.

Many studies have been conducted on chemokines and their receptors in hepatocellular carcinoma (HCC). However, various axes may not play the same role in HCC as they do in other tumors. Currently, chemokines and their receptors such as the CXCL12-CXCR4 axis and the CCL20-CCR6 axis have received much research attention. This paper will analyze the relationship between chemokines and their receptors in HCC.

CXCL12-CXCR4 AXIS IN HCC

The CXCL12-CXCR4 axis is considered to be an important factor in the regulation of angiogenesis which is essential for growth and progression of HCC. Using immunohistochemistry, Li *et al*[6] found a much higher expression of the CXCL12-CXCR4 axis in HCC specimens than in adjacent, cirrhosis, liver adenocarcinoma, and normal liver tissues. The findings of the study by Sutton *et al*[7] indicated that the CXCL12-CXCR4 axis in HCC cell lines promoted the growth, invasion and metastasis of these cell lines. Liu *et al*[8] suggested that CXCR4 and CXCL12 may play an important role in the metastasis of HCC by promoting the migration of tumor cells. In addition, some researchers found that fucoidan exhibited antitumor activity toward Huh7 cells through the down-regulation of CXCL12 expression[9]. Data from recent research strongly suggests that CXCL12-CXCR4 stimulates the proliferation of oval cells, and HCC occurrence correlates with the abnormal differentiation of these cells[10]. All these studies reveal a strong association between CXCL12-CXCR4 and the incidence of HCC development, however, the exact mechanism remains unknown. Chu *et al*[11] conducted studies on HCC cell lines which suggested that the CXCL12-CXCR4 system mediates active MMP-9 and MMP-2 secretion, which facilitates the consequent metastasis of those cells. Schimanski *et al*[12] found that in patients with HCC, the expression of CXCR4 significantly correlated with the progression of local tumor, distant dissemination of lymphatic metastasis, as well as a decreased 3-year-survival rate. Strong expression of CXCR4 was significantly associated with HCC progression. They also found that loss of p53 function did not impact on CXCR4 expression, which indicated that CXCR4 influences HCC progression through other channels. However, Schimanski's work did not suggest which channel may be involved.

Some researchers have shed doubt on whether CXCL12-CXCR4 promotes the growth and progression of HCC. Using immunohistochemistry and RT-PCR, Shibuta *et al*[13] detected the down-regulation of CXCL12/CXCR4 expression in HCC and also found that CXCR4 expression correlated with the cell cycle when studying HCC cell lines. Nahon[14] conducted a retrospective analysis on 120 HCV-infected HCC patients, and suggested a lack of association between CXCL12/CXCR4, death and HCC recurrence. New research shows that SDF-1-3'A (CXCL12) gene polymorphisms are considered to be relevant factors in HCC occurrence and development which have little to do with CXCR4[15]. Different findings from previous studies can be attributed to the various research methods and statistical methods adopted. These studies need to be improved. For example, in Nahon's study, results could be achieved if the sample size was increased. In addition, most of the HCC studies did not include different forms of hepatitis (HBV and HCV) as a variable.

Therefore, even though most of the research data show that the CXCL12-CXCR4 axis is important in HCC, its specific role is still unknown and some evidence to the contrary can not be explained. Further study and discussion are needed to clarify these issues.

CCL20-CCR6 AXIS IN HCC

There is currently an increasing interest in the CCL20-CCR6 axis in HCC. Fujii *et al*[16] found in *in vitro* experiments that the CCL20-CCR6 axis may promote the growth of the hepatoma cell line Huh7 through phosphorylation of mitogen-activated protein kinase (MAPK).

Rubie *et al*[17] also studied CCL19, CCL20, CCL21, CXCL12 and the expression of their receptors in HCC, and found that all chemokines were found to be expressed in normal liver and HCC tissues, yet CCL20 was the only chemokine which showed significant up-regulation in HCC tissues. The only chemokine receptor to show significant up-regulation in HCC tissues was CCR6. Clinicopathological analysis revealed a strong association between the levels of expression and the degrees of differentiation. That is, with high differentiation, the expression was low. Therefore, the authors proposed that the CCL20-CCR6 axis plays an important role in the growth and progression of HCC, namely a distinct increase in CCL20 expression rates in HCC tis-

sues of grade Ⅲ tumors in comparison to grade Ⅱ tumors. In addition, high expression of CCL20 is often accompanied by high expression of CCR6. Therefore, the authors suggested that CCL20-CCR6 may be involved in the formation and development of HCC.

Uchida *et al*[18] observed the formation of pseudopodia in HCC cell lines with high expression of CCR6, which was not observed in cells low in CCR6. The incidence of intrahepatic metastasis was higher in patients with increased expression of CCR6 than in patients with low expression of CCR6. Disease-free survival was significantly poorer in the high CCR6 expression group than in the low CCR6 expression group. The author concluded that CCR6 might be associated with the intrahepatic metastasis of HCC and may be used as a prognostic factor after hepatic resection for HCC.

In addition, researchers have studied the differences in RNA and protein expression of the chemokines CXCL12, CCL19, CCL20, CCL21 and their receptors in primary liver cancer and colon cancer metastasis; even though they have a high expression of CCL20 and CXCR4, the expression in colon cancer liver metastasis was significantly higher than that in primary liver cancer, thus CXCR4 is important in differentiating primary and secondary liver tumors, while CCL20 and its receptor CCR6 may promote tumor growth in addition to their role in identifying primary and secondary liver tumors[19].

Although a large number of studies show that the CCL20-CCR6 axis is strongly associated with HCC, the exact mechanism by which the CCL20-CCR6 axis promotes the growth and progression of HCC remains unknown.

FRACTALKINE-CX3CR1 AXIS IN HCC

Fractalkine (CX3CL1) and its receptor CX3CR1 are expressed in DC, NK cells, CD8+ T cells, and macrophages. Although studies have shown that fractalkine can regulate the host immune response, the relationships between the fractalkine-CX3CR1 axis, tumor biochemistry and HCC are unclear. According to recent studies, the fractalkine-CX3CR1 axis is critical in the diagnosis of HCC, because it can not only regulate the immune response, but can also regulate the cell cycle of HCC[20]. Relevant experiments indicate that fractalkine can enhance the anti-tumor effect of the immune system against HCC in mice. Tang *et al*[21] observed that CX3CL1 could elicit tumor-specific cytotoxic T cells and an increased production of IL-2 and IFN-γ capable of inhibiting tumor growth. Matsubara *et al*[20] found that HCC patients with an high expression of fractalkine and its receptor CX3CR1 have a low rate of recurrence of intrahepatic metastasis or extrahepatic metastasis, which suggest that the expression of fractalkine and its receptor CX3CR1 may be related to the prognosis of HCC patients, and may be involved in tumor immunity by killing tumor cells.

However, some researchers have proposed that there is a lack of association between the CX3CL1-CX3CR1 axis and HCC, as studies have demonstrated that CX3CR1 is not a risk factor for HCC[22]. These researchers believe that tumor-derived chemokines have dual roles. Leukocyte aggregation following a signal of increasing chemokine concentration may not only be beneficial to the host, but also contribute to tumor growth. The roles virus-related chemokines play in tumors may be multiple, therefore the specific role of CX3CR1 in the formation of chronic hepatitis and liver disease needs to be elaborated.

In addition, the up-regulated expression of CX3CL1-CX3CR1 in ischemia-reperfusion injury suggests that CX3CL1-CX3CR1 is crucial in reperfusion injury[23].

OTHER RELATED CHEMOKINES IN HCC

Recent studies have found that the combination of CCL5 with the CCR1 receptor can promote metastasis and invasion of the HCC cell line Huh7[24]. Furthermore, studies of the expression of CCL3 and its receptor CCR1 in liver cancer have shown a significantly higher level of expression in cancer tissues. Animal experiments also confirmed the essential contribution of CCL3 and CCR1 to the growth and progression of HCC[25]. Recent studies indicated that CCL3 induced by IL-1 combined with its effective receptor CCR1 may be involved in the growth and progression of HCC. The authors studied six chemokines in six types of HCC cell lines and found that all the hepatoma cell lines constitutively and exclusively expressed CCR1 mRNA and its protein on their cell surface, however, the expression of CCR1 was not detected in normal liver cells[26]. This research suggested a close relationship between the CCL3-CCR1 axis and HCC.

VCC-1 (VEGF correlated chemokine-1) is a newly discovered chemokine, and has a high expression in breast and colon cancer. Studies have demonstrated that it promotes tumor growth by increasing angiogenesis[27]. Our recent studies have shown (findings are not yet published) that the expression of VCC-1 in cancer tissues of HCC patients is much higher than that in paracancerous tissues and normal liver tissues. This expression is related to tumor size and tumor differentiation. The findings suggest that VCC-1 may be involved in the growth and progression of HCC, but whether it plays a role in HCC recurrence and metastasis remains unknown. In addition, it is not clear with which receptor VCC-1 interacts.

CHEMOKINES AND THEIR RECEPTORS INVOLVED IN HCC TUMOR IMMUNITY

As mentioned above, not all chemokines promote tumor growth and metastasis. Fractalkine/CX3CL1 is believed to be able to inhibit tumor growth[21]. Hirano *et al*[28], discovered that some chemokines induced by IFN-γ such as Mig (CXCL9) and IP-10 (CXCL10) can promote the gathering of lymphocytes in HCC, and are essential in tumor immunity.

Despite lymphocyte infiltration in liver tumors, the tumor grows and spreads rapidly. Liu *et al*[29] studied the expression of chemokines in HCC tissues and HCC cell lines, and found that IP-10 and Mig were expressed in

serum and tissues. The expression of IP-10 in cancer tissues was higher than that in paracancerous tissues. Autocrine chemokines in HCC cell lines can lead to CXCR3-specific lymphocytes infiltration. However, if the liver cancer cell lines and normal lymphocytes are cultivated, the expression of CXCR3 in lymphocytes shows a distinct decrease. As a result, the combination of CXCR3 and IP-10 and metastasis of T cells to tumor cells are both reduced. *In vitro* experiments show weakening chemotactic effects of CD4+ and CD8+ T cells from liver cancer cells on IP-10, whereas the chemotactic effects of T-cells from normal tissues on IP-10 were enhanced. This study demonstrated the functional desensitization of the chemokine receptor CXCR3 in lymphocytes from HCC patients by CXCR3 ligands secreted by tumor cells. This may cause lymphocyte dysfunction and subsequently impaired immune defense against the tumor.

CONCLUSION

In summary, the roles of chemokines in HCC are multiple, and range from promoting to inhibiting. But which role is dominant? HCC may be regulated by complicated chemokines and their receptors. Current studies show that in tumors such as lung cancer, colon cancer and prostate cancer, the CXCL12-CXCR4 axis may be related to tumor metastasis of distant organs and the CCL21-CCR7 axis may be associated with lymph node metastasis. However, further study is needed to clarify these associations in HCC. In addition, there are few reports on the expression of the CCL21-CCR7 axis in HCC although much research has been carried out on the relationship of this axis with lymph node metastasis in other tumors.

Researchers are expected to explore the possibility of chemokines and their receptors being target genes and to utilize their anti-tumor function when treating HCC. Recent studies have shown that co-expression of suicide genes and MCP-1 (monocyte chemotactic protein-1) in rat liver and colon cancer has a significant anti-tumor effect[30]. Gene therapy based on a variety of chemokine axes such as the CXCL12-CXCR4 axis is being studied. The desired effect can only be achieved by combining several chemokine axes rather than focusing on a single axis.

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ORIGINAL ARTICLE

Role of HSP-90 for increased nNOS-mediated vasodilation in mesenteric arteries in portal hypertension

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Abstract

AIM: To explore the role of heat shock protein-90 (HSP-90) for nitrergic vasorelaxation in the splanchnic circulation in rats with and without portal hypertension.

METHODS: Neuronal nitric oxide synthase (nNOS) and HSP-90 were analyzed by immunofluorescence, western blotting and co-immunoprecipitation in the mesenteric vasculature and isolated nerves of portal-vein-ligated (PVL) rats and sham operated rats. *In vitro* perfused de-endothelialized mesenteric arterial vasculature was preconstricted with norepinephrine (EC_{80}) and tested for nNOS-mediated vasorelaxation by periarterial nerve stimulation (PNS, 2-12 Hz, 45V) before and after incubation with geldanamycin (specific inhibitor of HSP-90 signalling, 3 μg/mL) or L-NAME (non-specific NOS-blocker, 10^{-4} mol/L).

RESULTS: nNOS and HSP-90 expression was significantly increased in mesenteric nerves from PVL as compared to sham rats. Moreover, nNOS and HSP-90 were visualized in mesenteric nerves by immunofluorescence and immunoprecipitation of nNOS co-immunoprecitated HSP-90 in sham and PVL rats. PNS induced a frequency-dependent vasorelaxation which was more pronounced in PVL as compared to sham rats. L-NAME and geldanamycin markedly reduced nNOS-mediated vasorelaxation abrogating differences between the study groups. The effect of L-NAME and geldanamycin on nNOS-mediated vasorelaxation was significantly greater in PVL than in sham animals. However, no difference in magnitude of effect between L-NAME and geldanamycin was noted.

CONCLUSION: HSP-90 acts as a signalling mediator of nNOS-dependent nerve mediated vascular responses in mesenteric arteries, and the increased nitrergic vasorelaxation observed in portal hypertension is mediated largely by HSP-90.

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Key words: Heat shock protein-90; Nitric oxide; Vasodilation; Portal hypertension; Mesenteric circulation

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INTRODUCTION

Arterial vasodilation and vascular dysregulation represent the initiating as well as perpetuating mechanism in the pathophysiology of the hyperdynamic circulatory syndrome in portal hypertension[1]. This arterial vasodilation occurs early and is most pronounced in the splanchnic circulation which therefore has been suggested to be the main culprit for reduced peripheral vascular resistance occurring with progression of portal hypertension[2,3]. A strong line of evidence suggests that vascular overproduction of nitric oxide (NO) plays a pivotal role for the development of splanchnic arterial vasodilation in this condition[4-6]. In fact, non-specific inhibition of NO synthesis has been shown to restore mesenteric vascular responsiveness[7] and to almost normalize splanchnic vascular resistance[8] thereby markedly ameliorating portal-systemic shunting and the hyperdynamic circulation[9,10].

Three isoforms of NO-synthases (NOS) have been cloned. Vascular overproduction in the splanchnic circulation in portal hypertension is derived from endothelial (eNOS) and neuronal (nNOS) but not inducible NOS (iNOS)[4,11,12]. eNOS as well as nNOS-derived NO production are regulated through distinct posttranslational modifications, one being the interaction of NOS with regulatory proteins[13]. The molecular chaperone heat shock protein-90 (HSP-90) facilitates folding and stabilization of cellular proteins thereby promoting specific signalling pathways[14]. In support of this concept, interaction of eNOS and nNOS with HSP-90 has recently been shown to facilitate NO synthesis[15,16]. In fact, in portal hypertension we have observed increased eNOS activity and an associated enhanced eNOS-dependent vasorelaxation in mesenteric arteries, which was markedly inhibited by geldanamycin, an ansamycin antibiotic and specific HSP-90 antagonist[17]. Whether HSP-90 likewise plays a crucial role in nNOS-mediated vasodilation in the splanchnic circulation in healthy as well as portal hypertensive conditions is currently unknown.

Chronic specific nNOS-inhibition markedly attenuates the hyperdynamic circulatory syndrome in experimental cirrhosis[18] underlining the relative importance of nNOS-derived NO in this scenario. Moreover, we recently reported an increased nNOS expression in the whole mesenteric vascular bed of portal vein-ligated (PVL) rats[12], and nNOS has been demonstrated to be up-regulated and to be highly expressed in vascular smooth muscle cells of the mesenteric arteries during portal hypertension[19]. nNOS expression and function in mesenteric nervous tissue independent of the arterial smooth muscle layer, however, has not been investigated so far.

Thus, the aim of our study was to analyse (1) nNOS expression in mesenteric nerves during portal hypertension; (2) whether HSP-90 co-localizes with and regulates nNOS-mediated vasorelaxation in the mesenteric arterial vasculature; and (3) if this is the case, whether alterations in HSP-90 function occur during portal hypertension.

MATERIALS AND METHODS

Animals

The investigation was performed in male Sprague-Dawley rats (Harlan Sprague Dawley Laboratories, Indianapolis, IN), weighing 300-399 g. Rats were caged at a constant room temperature of 21℃, exposed to a 12:12 hour light: dark cycle, and allowed free access to water and standard rat chow *ad libitum*. All experimental procedures in this study were conducted according to the German Physiological Society principles for the care and use of laboratory animals (Granted permission number 621-2531.1-23/00, Government of Oberpfalz, Bavaria).

Drugs

Acetylcholine, Sodium-Nitroprusside, Norepinephrine, Guanethidine, Atropine, Timolol, L-NAME and geldanamycin were purchased from Sigma (Deisenhofen, Germany).

Induction of portal hypertension

A prehepatic portal hypertensive animal model extensively studied in our laboratory was used[20]. Portal hypertension was induced surgically in aseptic conditions. Briefly, the rats were anaesthetized with ketamine hydrochloride (Ketalar, 100 mg/kg bw; Parke, Davis, Avon, CT). After a midline abdominal incision, the portal vein was freed from surrounding tissue. A ligature (silk gut 3-0) was placed around a 20-gauge blunt-tipped needle lying alongside the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein. In sham-operated rats, the same operation was performed with the exception that after isolating the portal vein no ligature was placed. All studies were performed in 12-18 h fasted animals 10-14 d after surgery.

In vitro perfusion

The *in vitro* perfusion system used was a partial modification of that originally described by McGregor and used extensively in previous studies from our laboratory and others[5,21,22]. Briefly, the superior mesenteric artery (SMA) was cannulated with a PE-60 catheter and gently perfused with 15 mL warm Krebs solution to eliminate blood. After isolating the SMA with its mesentery, the gut was cut off close to its mesenteric border. The arterial vasculature was then transferred to a 37℃ water-jacketed container and perfused with oxygenated 37℃ Krebs' solution (95% O_2, 5% CO_2) using a roller pump (Ismatec, IPC 8-channel, Zürich, Switzerland). The Krebs solution had the following composition (mmol/L): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; disodium EDTA, 0.026; and glucose, 11.0; pH 7.4. The effluent of the perfused tissue was continuously removed from the perfusing chamber. The perfusion pressure was measured with a P-23-Db strain gauge transducer (Statham, Oxnard, CA) on a side arm just before the perfusing cannula and continuously

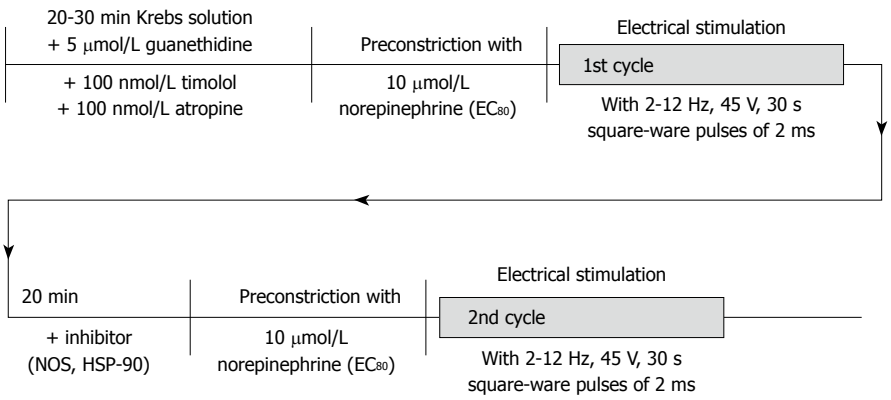

Figure 1 Experimental study protocol. In this illustration, the chronological order of applications of drugs and performance of peri-arterial nerve stimulation (PNS) is shown. EC_{80}: Concentration achieving 80% maximal vasoconstriction; NOS: Nitric oxide synthase; HSP-90: Heat shock protein-90.

recorded (Powerlab Quadbridge and Powerlab 4/20, from AD Instruments, Spechbach, Germany).

Where indicated endothelial denudation of the mesenteric vasculature was performed by a combined treatment of cholic acid (sodium salt) and distilled water as has been used before[21]. In brief, after cannulation of the SMA and gentle flushing with 10 mL of warmed Krebs' solution to eliminate blood, perfusion with cholic acid (0.5%/1.5 mL for 10 s) followed by 15 mL of Krebs solution (to eliminate cholic acid) was performed. The preparation was then transferred to the 37℃ waterjacketed container and perfused with oxygenated 37℃ Krebs solution (4 mL/min) for 10 min. After the mesenteric vasculature was relaxed, 37℃ warmed distilled water was perfused for 10 min before starting Krebs perfusion again. After an equilibration period of 45 min, experimental perfusion protocols were performed (Figure 1). At the end of each experiment we assessed whether the vessel was completely de-endothelialized and whether the smooth muscle function was maintained. The mesenteric preparation was kept preconstricted with methoxamine (MT; α-1-agonist: 100 μmol/L) and dose-dependent vasorelaxation to the endothelium-dependent vasodilator acetylcholine (ACh: 10^{-8} to 10^{-6} g, bolus of 0.1 mL) and the endothelium-independent vasodilator sodium nitroprusside (SNP: 10^{-6} to 10^{-5} g, bolus of 0.1 mL) was tested. Only experiments with ACh-/ and SNP-induced relaxation being less/more than 15%/60% were accounted as valid.

Periarterial nerve stimulation

Two platinum electrodes, one placed around the SMA and the other shaped as a wire grid the tissue was resting on, were used for transmural electrical field stimulation. The nerves of the preparation were stimulated by means of an electronic stimulator (I-ZQ4v, Hugo Sachs Electronics, Hugstetten, Germany) , delivering single square-wave pulses (2 ms) at 45 V with a train duration of 30 s and frequencies of 2-12 Hz. In order to evaluate vasodilatory responsiveness vessels were preconstricted submaximally (EC_{80}) using norepinephrine (NE 10^{-5} mol/L) before applying periarterial nerve stimulation (PNS) (Figure 1). nNOS-mediated vasorelaxation is known to be non-adrenergic and non-cholinergic in origin. Therefore, guanethidine (5×10^{-5} mol/L), atropine (10^{-9} mol/L) and timolol (10^{-9} mol/L) were added from the beginning in order to deplete endogenous noradrenaline stores and to prevent its uptake as well as to avoid cholinergic stimulation. When a stable preconstriction level was achieved, PNS was applied in a non-cumulative fashion. Sufficient time was allowed between each stimulation train for the perfusion pressure to return to a stable level, usually 5-10 min. PNS responses are expressed as percentage change of the pre-constriction level being present before PNS. Nitrergic vasodilation is known to be independent of prostaglandin synthesis[23] and it is not subject to pre-junctional inhibition by alpha2-adrenoreceptors[23]. Moreover, in previous experiments tetrodotoxin has been used to evidence the neural origin of the hemodynamic response[12].

Role of HSP-90 for nNOS-mediated vasorelaxation

Most importantly, all PNS experiments were performed in de-endothelialized mesenteric vasculature in order to exclude any hemodynamic effects of endothelium-derived vasodilators. In order to evaluate the magnitude of nNOS-mediated vasorelaxation, PNS experiments were performed in the absence (1st perfusion cycle) and presence of L-NAME (10 μmol/L) (2nd perfusion cycle after incubation with L-NAME for 20 min). The differences in PNS-triggered vasorelaxation induced by L-NAME correspond to nNOS-mediated vasodilator effects. As for the role of HSP-90 in mediating this nNOS-vasodilation, vessel preparations were correspondingly pre-incubated with the HSP-90 inhibitor geldanamycin (GA 3 μg/mL) for 20 min before performing the 2nd perfusion cycle (Figure 1). GA is a benzoquinone ansamycin antibiotic that binds to the nucleotide-binding site of HSP-90 and specifically blocks HSP-90 function[24,25]. GA was first reported to inhibit iNOS activity in rat smooth muscle[26], however, as reported earlier no iNOS protein was detected in the vasculature studied, ruling out a hemodynamic role of HSP-90 related to iNOS-derived NO. As shown previously, incubation with GA does not directly affect soluble guanylate cyclase or other smooth muscle cell machinery required for NO-dependent vasodilation since vasodilation is not altered in response to SNP[17].

Immunofluorescence analysis

Mesenteric tissues were harvested by dissecting and removing the highly vascular tissue situated between the mesenteric lymph nodes and small intestine. The tissue was freed, washed in PBS, stored in 3.7% formaldehyde at 4℃ over night, and then transferred into 20% sucrose solution

for more than 12 h at 4℃. After this procedure the SMA were embedded in tissue tek (Herstetller, Stadt, Land) and stored at -80℃. Frozen sections (Frigocut 2800 E, Leica, Wetzlar, Germany) were incubated overnight with monoclonal antibodies directed against nNOS or HSP-90, respectively (both BA, Transduction Laboratory, San Diego, CA, USA). Immunofluorescence staining was performed by utilizing either red fluorescent or green fluorescent anti-mouse IgG (Alexa 46, MoBiTec, Göttingen, Germany) as secondary antibodies. Sections treated with secondary antibody only or with control IgG (as first antibody) did not reveal an immunosignal (data not shown).

Immunoprecipitation and Western blotting

Mesenteric nerves located in near proximity to the SMA were dissected microscopically and washed in PBS and homogenized in a lysis buffer. Protein quantification of tissue samples was performed using the Lowry assay. In experiments examining the level of nNOS and HSP-90 protein in mesenteric tissue, 100 μg of protein were used for electrophoresis. For nNOS immunoprecipitation, about 1500 μg of detergent-soluble protein was incubated with excess nNOS mAb overnight after samples were precleared with Pansorbin (Calbiochem, CA, USA). Immune complexes were precipitated by the addition of protein A-Sepharose. Protein samples were boiled in Laemmli loading buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% acrylamide gel, and proteins were electroblotted onto nitrocellulose membranes. Subsequently, the membranes were washed with Tris-buffer saline with 1% Tween, blocked with 5% milk, and incubated with either HSP-90 mAb or nNOS mAb. Densitometry was performed with a scanner and analyzed with the Software Image Quant (Personal Densitometer Si, Amersham, Bioscience, UK).

Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed using ANOVA (two-way, with repeated measurements) for comparison of study groups for PNS-induced vasorelaxation. Unpaired non-parametric tests were used for comparison of individual values between study groups. The level of statistical significance was set at $P < 0.05$.

RESULTS

Animals

There were no differences in body weight in the experimental groups (349 ± 36 g for PVL and 363 ± 42 g for sham rats, respectively). PVL rats showed elevated spleen weights, expressed in relation to body weight (PVL: 3.25 ± 0.30 g/kg bw *vs* sham: 2.48 ± 0.50 g/kg bw, $P < 0.0001$).

Western blotting and co-association of nNOS and HSP-90 in mesenteric nerves

To determine the importance of HSP-90 for neuronal vascular control in mesenteric arteries, we first examined expression, association and localization of nNOS and HSP-90 in mesenteric nerves. nNOS expression was found to be increased in PVL rats as compared to sham rats (Figure 2A and C). In addition, HSP-90 expression was enhanced in mesenteric nerves in PVL rats as compared to sham rats (Figure 2B and C) and nNOS co-precipitated with HSP-90 from mesenteric nerves (Figure 3) evidencing direct protein-protein interaction.

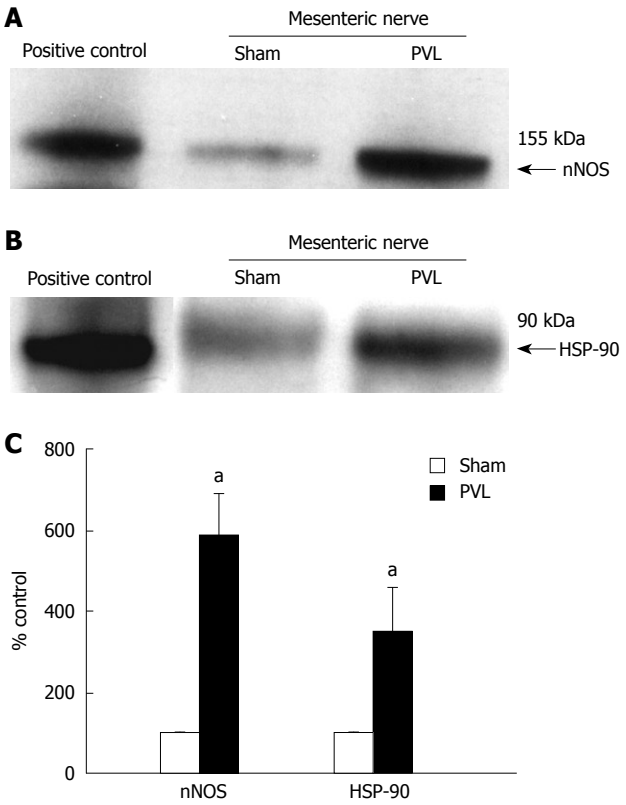

Figure 2 n-nitric oxide synthase (nNOS) and HSP-90 expression in mesenteric nerves. nNOS (A) and HSP-90 (B) in mesenteric nerves of portal-vein-ligated rats (PVL) and sham rats by Western blotting analysis. Protein lysate from rat brain tissue was used as positive control; C: Densitometric analysis of Western blottings revealed a more than 5-fold increase of nNOS and approximately 3-fold increase of HSP-90 protein expression in mesenteric nerves of PVL rats as compared to sham rats. $^aP < 0.05$, $n = 3$ per group.

Immunofluoresence of nNOS and HSP-90 in mesenteric nerves

We previously reported that both nNOS and HSP-90 are abundantly expressed in mesenteric vasculature in rats[17]. Here we extend this information with a focus on the mesenteric neurons. In fact, immunofluorescence revealed co-localization, with nNOS and HSP-90 being distributed equally in perivascular neurons (Figure 4A and B). Indeed, identical localization and most obviously similar magnitude of protein expression for nNOS as well as HSP-90 were observed at the entry of the nerve supply into the mesenteric vasculature.

nNOS-dependent mesenteric vasorelaxation and HSP-90

Baseline perfusion pressures before removal of the endothelium were significantly lower in PVL as compared to sham rats (16.0 ± 6.1 mmHg *vs* 23.4 ± 8.0 mmHg, $P < 0.01$). After removal of the endothelium no difference in basal perfusion pressure between study groups was noted anymore (data not shown). The vasodilator response to

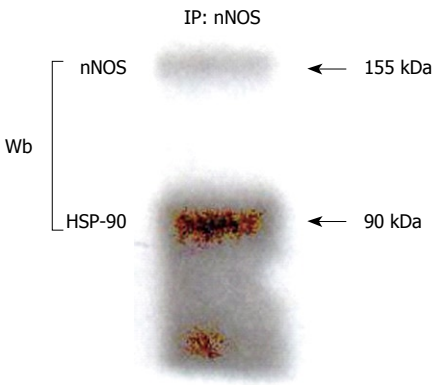

Figure 3 Co-immunoprecipitation. nNOS was immunoprecipitated from detergent-soluble protein lysates prepared from de-endothelialized mesenteric tissue harvested as described in materials and methods and prepared for gel electrophoresis or, alternatively, protein samples were directly prepared for gel electrophoresis. nNOS and HSP-90 are both abundantly expressed in mesenteric tissue and immunoprecipitation (IP) of nNOS coprecipitates HSP-90 under basal conditions. Wb: Western blotting.

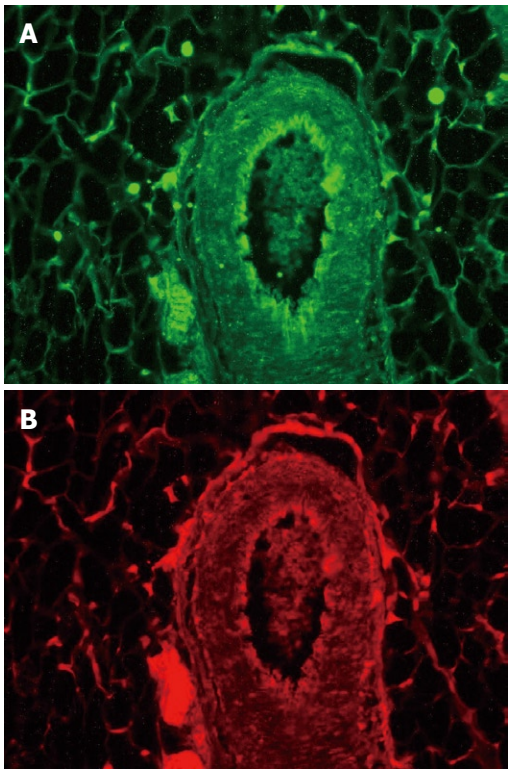

Figure 4 Immunofluoresence analysis of nNOS and HSP-90. As can be seen immunofluorescence shows strong signals for nNOS (A) and HSP-90 (B) mainly at the entry of the nerve bundle along the adventitia revealing identical localization of nNOS and HSP-90 protein in mesenteric nervous tissue.

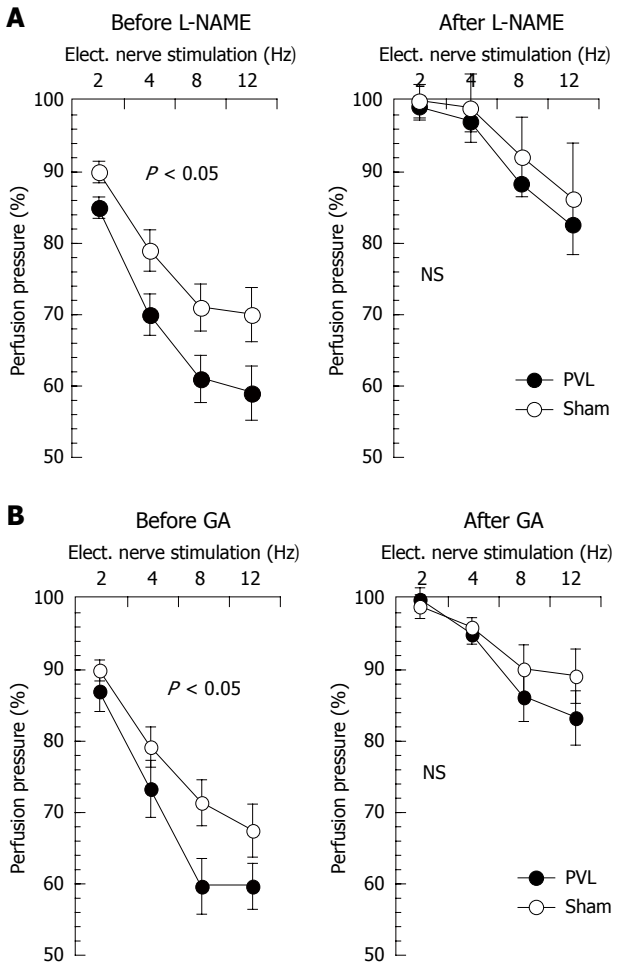

Figure 5 nNOS- and HSP-90-mediated neuronal vasorelaxation in PVL and sham rats. PNS-induced nitrergic vasorelaxation in de-endothelialized mesenteric vasculature is depicted before and after L-NAME (A) as well as before and after HSP-90-inhibition with geldanamycin (B). It can be appreciated that no more significant differences in frequency-dependent neuronal vasorelaxation were observed between the study groups after L-NAME as well as after geldanamycin. Values given as mean ± SE. PVL-group: $n = 6$; Sham group: $n = 6$. $P < 0.05$ for ANOVA comparing study groups. Elect. nerve stimulation: Electric nerve stimulation; NS: Not significant.

acetylcholine at the highest dose used was 7.5% ± 5.0% and 9.0% ± 6.0% in PVL and sham rats, respectively (NS), demonstrating a sufficient de-endothelialization. The vasodilator response to sodium nitroprusside at the highest dose applied was 65% ± 7% for PVL and 58% ± 9% for sham rats (NS) demonstrating the functional integrity of the vascular smooth muscle cells in the vascular bed studied. NE-induced pre-constriction levels were similar in both study groups (95 ± 5 mmHg *vs* 88 ± 6 mmHg) indicating similar vascular tone before neuronal stimulation. Incubation with either L-NAME or GA did not affect baseline or pre-constriction levels in either group. All vessels responded to PNS with a frequency-dependent decrease in perfusion pressure (Figure 5). This neurally mediated vasodilatory response was significantly more pronounced in PVL as compared to sham rats (Figure 5). Non-specific NO-inhibition by L-NAME (Figure 5A) abolished the difference in PNS-induced vasorelaxation between the study groups evidencing NO as being the vasodilator mediating this difference and thus reflecting nNOS-mediated vasorelaxation. Moreover, HSP-90 inhibition using GA likewise abolished the difference in PNS-induced vasorelaxation between PVL and sham rats (Figure 5B). This indicates that the observed increase in nNOS-mediated vasorelaxation in PVL rats is dependent on HSP-90 signalling.

When expressing PNS-induced vasorelaxation as percent change from the pre-constriction level a markedly increased response was observed in vessel preparations from

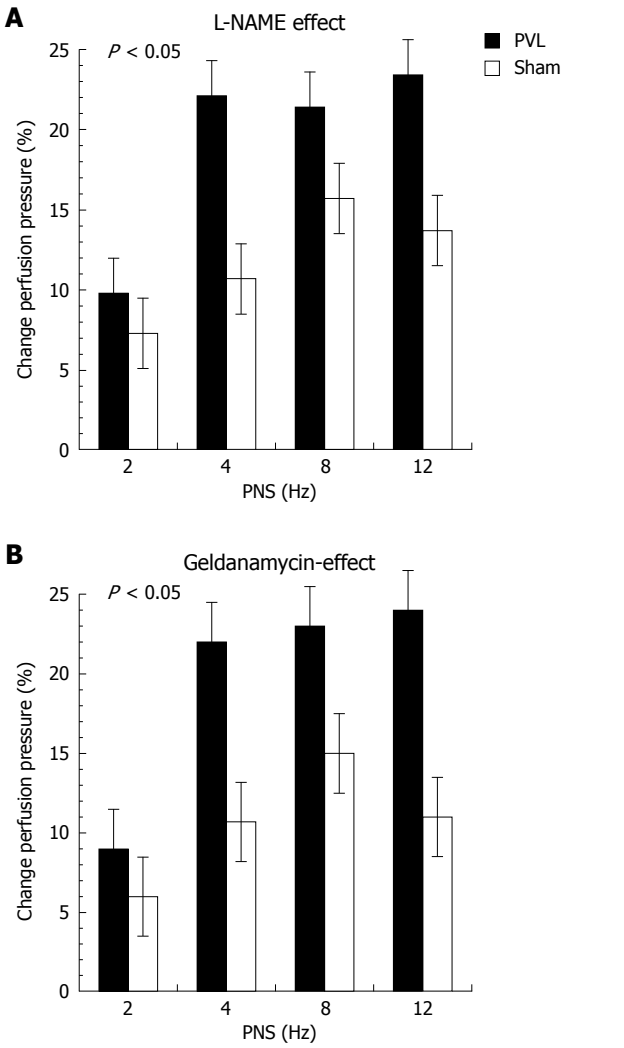

Figure 6 Changes in PNS-induced vasorelaxation induced by inhibition of NOS or HSP-90. Changes in pressure response to PNS induced by L-NAME (A) and geldanamycin (B) presented as percent change in perfusion pressure as compared to values obtained during the first perfusion cycle. $P < 0.05$ (ANOVA comparing all conditions between study groups) *vs* sham, respectively.

PVL rats as compared to sham rats (Figure 6). Maximal vasodilation was reached at 8-12 Hz, confirming previous investigations of SMA in rats[27]. Finally, the effect of NO inhibition (Figure 6A) and HSP-90 inhibition (Figure 6B) expressed as percent change in perfusion pressure from PNS-induced vasorelaxation observed during the corresponding 1st perfusion cycle induced by L-NAME or GA was significantly more pronounced in PVL rats as compared to sham rats ($P < 0.05$). In addition, the magnitude of effect induced by L-NAME and GA was almost identical indicating that the majority of nNOS-mediated vasorelaxation is dependent on HSP-90 signalling.

DISCUSSION

HSP-90 acts as an intermediate in the signalling cascade leading to activation of nNOS[28,29]. In this study, we examined the role of this protein interaction in mediating neuronal regulation of vascular tone in the mesenteric vascular bed, where vascular resistance contributes to the regulation of portal pressure. The novel findings of this study include the interaction of HSP-90 and nNOS in mesenteric nerves and the participation of this interaction in regulating vasomotor function in resistance vessels based on inhibition of HSP-90 with GA. Moreover, we demonstrate that after PVL, enhanced HSP-90 signalling contributes to the well-known elevated nNOS-dependent vasorelaxation.

Numerous investigations have presented evidence that nitrergic nerves are involved in vascular homeostasis[30,31]. However, mechanisms leading to nNOS-derived NO release from nitrergic nerve endings are missing. Under physiological conditions, NO production is strictly modulated to accommodate the need of homeostasis because abnormalites of NO formation cause disease[32,33]. Since NO is highly diffusable and cannot be stored in intracellular compartments modulation of NO production in cells is primarily mediated through regulating NOS activity. Several mechanisms have been revealed in NOS regulation. These include protein-protein interactions, protein phosphorylation, and subcellular targeting[34]. For example, regulation *via* protein-protein interaction such as HSP-90 and NOS appears particularly important. HSP-90 is one of the most abundant proteins in cells[35] constituting 1%-2% of total intracellular proteins. As a molecular chaperone, HSP-90 has been shown to regulate more than 100 signal transduction pathways by controlling function, trafficking and turnover of a variety of signalling proteins[36]. In respect to NOS regulation, HSP-90 was first reported to associate with eNOS acting as an allosteric enhancer[15]. Subsequent studies with purified nNOS preparations indicated that HSP-90 likewise functions as an allosteric enhancer for nNOS and inhibition of HSP-90 results in the inability to form the dimeric active nNOS[28]. Coupling of nNOS with HSP-90 is crucial for normal nNOS function[37]. Here, we report co-localization of HSP-90 and nNOS in mesenteric nerves. In addition, nNOS of mesenteric nerves co-immunoprecipitates with HSP-90 indicating protein-protein interaction in this neuronal tissue. Finally, GA was used to probe the importance of HSP-90 in cellular pathways, since it directly binds to the amino-terminal ATP binding domain of HSP-90[38] and specifically labels HSP-90 in cellular extracts[25]. Since NANC-induced vasorelaxation in the mesenteric vasculature is reduced after inhibition of HSP-90 by GA, the hemodynamic functional relevance of the stated protein-protein interaction is demonstrated. To our knowledge, this is the first report to demonstrate a physiological role of HSP-90 for regulation of neuronal control of vascular tone via modulation of nNOS-derived NO release. This is in accordance with previous reports demonstrating that HSP-90 inhibition causes a decrease of NO production in nNOS-transfected cells[16,39].

Portal hypertension is characterized by decreased systemic and particularly splanchnic vascular resistance which is the initiating mechanism for the development of the hyperdynamic circulatory syndrome in this entity[40]. Therefore, any measure improving splanchnic vascular resistance is of high hemodynamic and clinical relevance. Vascular overproduction of NO has been shown to be the pathophysiological hallmark leading to this splanchnic vasodilation and to be due to a marked upregulation of

eNOS and nNOS-derived NO synthesis[4-6,12]. Indeed, either non-specific or nNOS-specific NOS inhibition has been demonstrated to ameliorate the development of the hyperdynamic circulatory syndrome in experimental portal hypertension[9,18]. In addition, enhanced eNOS and nNOS protein expression as well as an increased vasodilatory response to eNOS and nNOS stimulation has been reported by us and others in the mesenteric vasculature during experimental portal hypertension[5,12,41]. Beside AKT phosphorylation[42], a marked contributory role of HSP signalling for eNOS-derived NO overproduction has been reported by us previously[17]. Here, we show that HSP-90 also largely mediates the enhancement in nNOS-dependent NO release in the mesenteric vasculature of pre-hepatic portal hypertensive rats. This is based on the observation of enhanced HSP-90 expression in mesenteric nerves of portal hypertensive rats and the finding that inhibition of HSP-90 signalling preferentially blocks NANC vasorelaxation in these animals.

Enhanced HSP-90 protein expression has been reported previously in whole mesenteric tissue of portal vein ligated rats[43]. However, evaluation of HSP-90 expression in mesenteric nerves has not been reported before. Nonetheless, it has been evidenced in cell lines as well as in studies with purified nNOS that HSP-90 directly augments NO synthesis of nNOS in a dose-dependent manner[16,37,44]. This effect has been attributed mainly to an increase in binding of calmodulin to nNOS mediated by HSP-90[44]. By this mode of action, most importantly HSP-90 treatment has been shown to increase even the maximal nNOS activity and associated maximal nNOS-dependent NO synthesis achievable[44]. Therefore, we propose that increased HSP-90 protein expression observed in mesenteric nerves of portal hypertensive rats results in enhanced nNOS-mediated vasorelaxation in the mesenteric vasculature of these animals due to its well-known augmenting effect on the nNOS-machinery. However, neither inhibition of NO nor HSP-90 did completely abrogate NANC vasorelaxation. Remaining vasodilation may well be mediated by other endogenous neurogenic vasodilators such as calcitonin-gene-related peptide or vasoactive-intestinal peptide known to be released from NANC nerves, particularly in rats. Nonetheless, these mediators are not mediating any differences in NANC-vasodilatory responses between the study groups since no significant difference in NANC vasorelaxation remained after NOS inhibition.

In summary, HSP-90 co-localizes with and binds to nNOS in mesenteric nerves playing a key role in nNOS-dependent vasoregulation in the mesenteric vasculature. This interaction serves to mediate the increased nitrergic vasorelaxation observed in the splanchnic circulation during experimental portal hypertension. In conjunction with our previous report on the excessive eNOS-derived NO overproduction depending mainly on HSP-90 signalling this study may provide the rational for pharmacological inhibition of HSP-90 as a novel target for the treatment of the hyperdynamic state in portal hypertension.

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COMMENTS

Background

Portal hypertension is the main cause of death in end-stage liver disease leading to multiple complications such as variceal hemorrhage, ascites and hepatorenal syndrome. Vasodilatation in the mesenteric circulation is of essential pathophysiological relevance in portal hypertension and n-nitric oxide synthase (nNOS) has gained much attraction as a vasodilation mediator in other clinical entities recently.

Research frontiers

Heat shock protein-90 (HSP-90) is a molecular chaperone required for the stability and activity of a diverse range of client proteins that have critical roles in signal transduction, cellular trafficking, cell growth, differentiation *etc.* but also vasoregulation. Among NOS it has been shown to be critical for full e- and nNOS activity. However, the role of HSP-90 in respect to neural nNOS-mediated vasoregulation has not been addressed in healthy or in portal hypertensive conditions. The strength of the current investigation relates to the utilization of the McGregor preparation enabling physiological and pharmacological testing of the whole mesenteric vasculature in its original anatomy and innervations. In contrast to arterial strips, this ensures testing of neural vasoregulation at close to *in vivo* conditions.

Innovations and breakthroughs

By this approach, pronounced nNOS-mediated vasodilation is confirmed in pre-hepatic portal hypertension and most strikingly is shown to be in large parts due to HSP-90 signalling. This is also the first time that HSP-90 has been established as a signaling mediator in neural vascular responses in healthy conditions.

Applications

Considering the well-known role of HSP-90 as an eNOS chaperone and enhanced eNOS-derived NO overproduction in portal hypertension, HSP-90 may well be an ideal target in treatment of portal hypertension.

Peer review

This is a good experimental investigation in which authors analyze the role of HSP-90 for neural vasoregulation in the mesenteric circulation in healthy and portal hypertensive conditions. The results are interesting and suggest that HSP-90 indeed is the main signaling intermediate translating into enhanced nNOS-dependent vasodilation during portal hypertension. Therefore, inhibition of HSP-90 could be used to treat portal hypertension.

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ORIGINAL ARTICLE

Different profiles of cytokine expression during mild and severe acute pancreatitis

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Abstract

AIM: To study secretion patterns of pro- and anti-inflammatory cytokines, and activation of various cellular subsets of leukocytes in peripheral blood.

METHODS: We have conducted a prospective observational study. One hundred and eight patients with a diagnosis of acute pancreatitis and onset of the disease within last 72 h were included in this study. The mRNA expression of 25 different types of cytokines in white blood cells was determined by quantitative real time polymerase chain reaction. Levels of 8 different cytokines in blood serum were measured by enzyme linked immunosorbent assay. Clinical data and cytokine expression results were subjected to statistical analysis.

RESULTS: Severe and necrotizing acute pancreatitis (AP) is characterized by the significant depletion of circulating lymphocytes. Severe acute pancreatitis is associated with a typical systemic inflammatory response syndrome and over-expression of pro-inflammatory cytokines [interleukin (IL)-6, IL-8, macrophage migration inhibitory factor (MIF)]. Serum IL-6 and MIF concentrations are the best discriminators of severe and necrotizing AP as well as possible fatal outcome during the early course of the disease.

CONCLUSION: Deregulation of cellular immune system is a key event leading to severe and necrotizing AP. IL-6 and MIF could be used as early predictors of complications.

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Key words: Acute pancreatitis; Cytokines; Prognostic factors

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INTRODUCTION

Incidence of acute pancreatitis (AP) is about 30-40 cases per 100 000 individuals, and it carries an overall mortality

rate of 10%-15%[1-3]. Mortality of patients with severe acute pancreatitis approaches 30%-40%[4-6]. Pancreatic necrosis develops early in the course of the disease and usually is well established by 96 h after the onset of clinical symptoms[7,8]. Both the risk of multiorgan failure (MOF) and infectious complications appear to be related to the degree of pancreatic necrosis[9]. If patients survive the critical early stages of systemic inflammatory response, development of septic complications is a major determinant of survival. Infected pancreatic necrosis and sepsis-related multiple organ failure account for up to 40%-50% of all deaths among AP patients[10-13].

At present it is widely accepted that the premature activation of digestive enzymes within pancreatic acinar cells is an initiating event that leads to the autodigestion of pancreas[14-16]. Once the disease process is initiated, common inflammatory and repair pathways are invoked. There is a local inflammatory reaction at the site of injury; if marked, this leads to a systemic inflammatory response syndrome (SIRS). An imbalance between the early SIRS, and the later compensatory counter-inflammatory response (CARS), and development of MOF are considered to be the primary causes of morbidity and mortality in severe acute pancreatitis. Excessive leukocyte activation (including neutrophils and monocyte-macrophage lineage) with cytokinemia plays a critical role in the pathogenesis of pancreatitis and even more so, of the subsequent inflammatory response[14-17]. It has been hypothesized that fatal pancreatitis is a consequence of abnormal phagocytic leukocyte hyperstimulation due to deregulation in T- and B-lymphocyte activation. However, the role of lymphocyte activation and its relation to the severity of disease in humans is still poorly understood[18-21]. Another important drawback, in our opinion, is that the majority of information about the alterations of the immune system during the AP comes from *in vitro* and *in vivo* studies, and therefore it is not always directly applicable and relevant to the clinical situation in human acute pancreatitis.

Several methods for estimating severity of acute pancreatitis are widely used today and include APACHE Ⅱ, Imrie and Ranson scores, the CT scoring system, and measurement of C-reactive protein and a number of laboratory markers[22-28]. Most of these multifactorial scores and laboratory tests are very good at identifying the critical situation, when MOF or local complication supervene, however, none can accurately predict the disease severity, development of pancreatic necrosis and/or possible fatal outcome during the first hours of hospitalization.

Thus, the aims of this study have been to investigate the secretion patterns of pro- and anti-inflammatory cytokines, and to estimate the activation of various cellular subsets of leukocytes (including lymphocytes, neutrophils, and monocyte-macrophage lineage) in peripheral blood of patients with severe and mild AP. Another goal of this study was to identify the serum soluble molecules that are associated with the development of severe acute pancreatitis, and which could be used as early markers to assess the local (pancreatic necrosis) and systemic complications (SIRS, MOF) later in the course of the disease.

MATERIALS AND METHODS

Study design and patient population

We have conducted a prospective observational study in the period between June 2005 and December 2007. All patients admitted to the Department of Surgery at Kaunas University of Medicine Hospital (Lithuania) with a diagnosis of acute pancreatitis and onset of the disease within last 72 h were included in this study (n = 108). The Regional Ethics Committee approved the study (protocols No. BE-2-47 and P1-113/2005) and all the patients provided written informed consent.

The diagnosis was established on the basis of acute abdominal pain, at least 3-fold elevated levels of serum amylase and typical radiological findings. A contrast-enhanced CT scan was performed on days 4 to 7 after onset of the disease to demonstrate the presence of pancreatic necrosis. According to the clinical course and clinical severity scores (APACHE Ⅱ > 7; Imrie-Glasgow > 2; MODS > 2), patients were stratified into mild and severe acute pancreatitis groups. Clinical data relating to the severity of disease, development of organ dysfunction and/or septic complications were prospectively collected in a standardized fashion. Patients with underlying chronic pancreatitis and patients with acute pancreatitis referred to our hospital from other institutions after management for more than 3 d were excluded from this study. Age- and sex-matched healthy subjects (n = 18) without previous medical history were enrolled as controls.

Peripheral blood samples from patients were drawn on admission to the hospital and, after centrifugation, samples were stored at -80℃ until analysis. The blood samples of control group underwent a similar process. Blood sample analysis was uniformly performed at the Laboratory for Molecular Research of Pancreas, Department of Surgery, Heidelberg University (Germany). The mRNA expression of 25 different types of cytokines in white blood cells (WBCs) was determined by quantitative real time polymerase chain reaction (QRT-PCR). Levels of 8 different cytokines in blood serum were measured by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Sigma-Aldrich GmbH, Steinheim, Germany).

Statistical analysis

Clinical data and cytokine expression results were used for statistical analysis. Statistical analysis was performed using SPSS® for Windows release 14.0 (SPSS, Chicago, Illinois, USA). Discriminant function analysis was used to determine differences between the groups. The diagnostic performance of a test, with a particular cut-off value, was established using Receiver Operating Characteristic (ROC) curve analysis. The data are presented as mean ± SE or median. For comparison between groups, the Mann-Whitney test or Student's t test were employed where appropriate. Results with $P < 0.05$ were considered statistically significant.

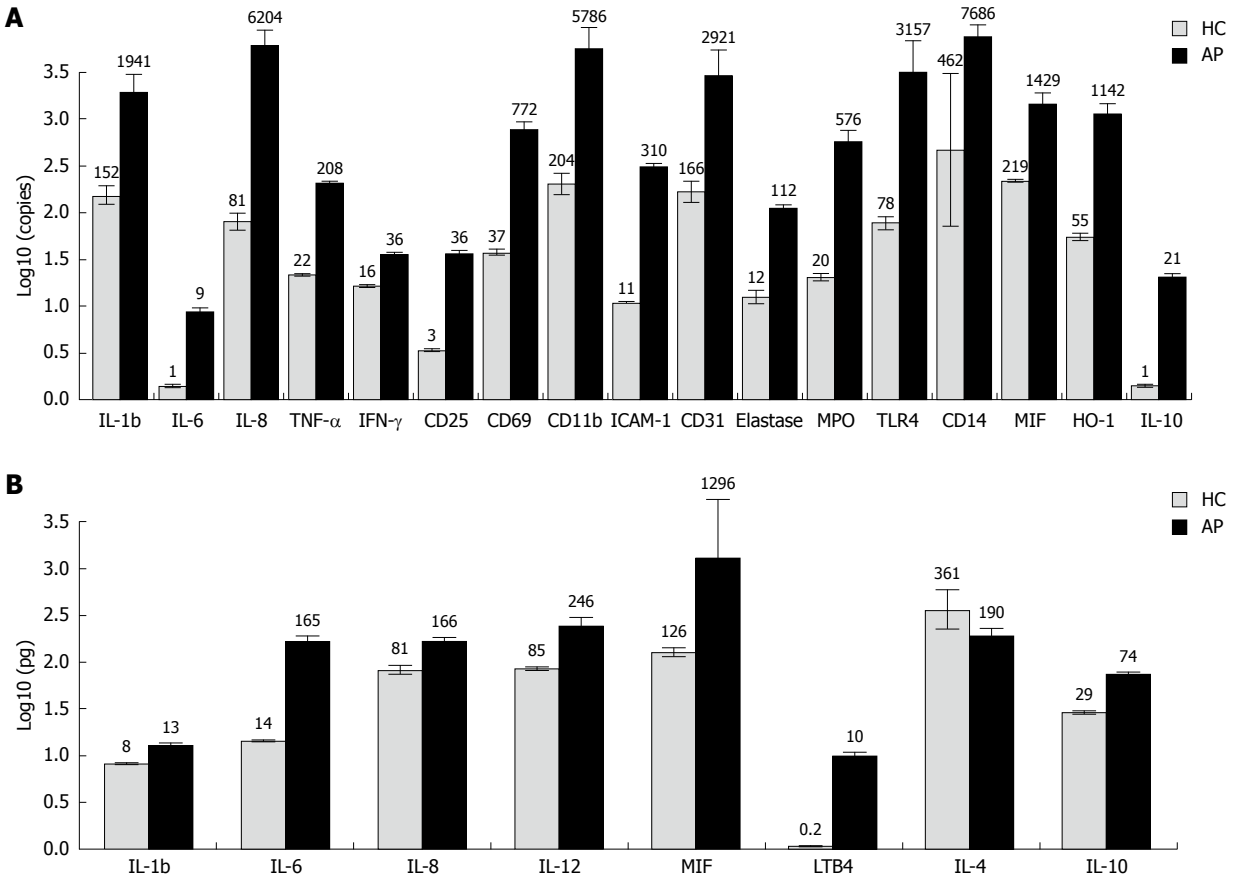


Figure 1 Cytokine expression at mRNA and protein level in peripheral blood of healthy subjects and acute pancreatitis (AP) patients. A: Gene expression in peripheral blood leukocytes of healthy subjects and AP patients. Gene expression at mRNA level of 25 different cytokines, adhesion molecules, lymphocyte activation markers, and other biologically active substances known to be linked to the inflammatory response were assessed by quantitative real time polymerase chain reaction (QRT-PCR) in peripheral blood leukocytes of AP patients and healthy controls. A median 18-fold (range: 2-77) increase in mRNA expression of pro-inflammatory molecules was observed in the AP group in comparison to healthy control subjects (HC). Activation of the anti-inflammatory system and some cytoprotective molecules was also notable in the AP patient group. Cytokine mRNA expression levels with statistically significant differences between AP and healthy control groups are shown; B: Cytokine concentration in blood serum of healthy controls and AP patients. Expression of eight different soluble molecules at protein level was assessed in serum of AP patients and healthy subjects using the enzyme linked immunosorbent assay (ELISA) method. A median 3-fold (range: 2-59) increase in protein expression of pro-inflammatory molecules was observed in the AP group when compared to HC. Cytokine expression levels with statistically significant differences between AP and healthy control groups are shown. IL: Interleukin; TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ; MPO: Myeloperoxidase; MIF: Macrophage migration inhibitory factor.

RESULTS

One hundred and eight patients (55 male and 53 female, mean age 54.1 ± 16.7 years) diagnosed with AP were included in the study. The main etiological factors of AP were alcohol (42.6%), high-fat diet (25.9%) and biliary stones (25.0%). Idiopathic pancreatitis was diagnosed in 7 cases (6.5%). Severe acute pancreatitis (APACHE Ⅱ > 7) comprised 44.4% of all cases. Necrotizing AP was detected by contrast-enhanced CT in 52 patients (48.1%), and high volume (> 30%) necrosis was present in 34 cases (31.3%). Multiple organ failure (> 2 systems) developed in 18 cases (16.7%). Overall mortality in our follow-up group was 12.9%; 9 patients (8.3%) died within first 2 wk of the disease and 5 patients (4.6%) later in the course of the disease. Eighteen healthy subjects (10 male and 8 female, mean age 40.1 ± 19.8 years) were studied as controls.

Gene expression at mRNA level of 25 different cytokines, adhesion molecules, lymphocyte activation markers, and other molecules known to be linked to the inflammatory response were assessed in peripheral blood leukocytes of AP patients and healthy controls. Results showed a typical pro-inflammatory cytokine profile in AP patients with an activation of different subsets of leukocytes (lymphocytes, monocyte-macrophage lineage and neutrophils). Activation of anti-inflammatory system and some cytoprotective molecules was also notable in the AP patient group (Figure 1A). A median 18-fold (range: 2-77) increase in mRNA expression of pro-inflammatory molecules was observed in the AP group in comparison to healthy controls. Expression of eight different soluble molecules at protein level was also assessed in peripheral blood serum of AP patients and healthy subjects using the ELISA method. Analysis revealed a typical over-expression of pro-inflammatory cytokines and altered expression of anti-inflammatory cytokines in AP patients compared to the healthy volunteers (Figure 1B). A median 3-fold (range: 2-59) increase in protein expression of pro-inflammatory molecules was observed in the AP group in comparison to healthy controls. Since many of these changes have been shown in other animal and clinical studies, this served as a

Table 1 Differences in clinical course of mild and severe acute pancreatitis (mean ± SD/count)

Clinical severity scores and patient characteristics	Mild AP	Severe AP	*P*
Apache Ⅱ (score)	3.6 ± 1.9	11.6 ± 4.7	< 0.01
Imrie-Glasgow (score)	2.2 ± 1.2	4.4 ± 1.5	< 0.01
MODS (score)	1.1 ± 1.3	4.3 ± 3.4	< 0.01
Necrotizing AP (count)	16	36	< 0.01
C-reactive protein (mg/L)	91.8 ± 18.7	152.9 ± 21.5	< 0.01
Necrosis volume > 30% (count)	8	26	< 0.01
Infectious complications (count)[1]	2	12	< 0.01
Presence of MOF (count)	0	18	< 0.01
Need for surgery (count)[2]	1	5	0.06
ICU stay (d)	1.0 ± 1.0	9.0 ± 2.0	< 0.05
Total hospital stay (d)	12.0 ± 1.0	19.0 ± 3.0	< 0.05
Number of early deaths (< 2 wk)	0	9	< 0.01
Number of late deaths (> 2 wk)	0	5	< 0.01

[1]Infectious complications: proven sepsis, infected pancreatic necrosis, lung and urinary infection; [2]Need for surgery: fasciotomy for intraabdominal compartment syndrome and/or necrosectomy. AP: Acute pancreatitis; MOF: Multiple organ failure; ICU: Intensive care unit.

control and proved that our methods function well.

For the next step of statistical analysis, AP patients were stratified into groups according to the severity of the disease based on presence of systemic (development of MOF) and local (pancreatic necrosis > 30%) complications, since these are the factors know to be associated with a poor outcome. Groups were formed on the basis of radiological findings on contrast-enhanced CT and APACHE Ⅱ clinical score, where all patients with score > 7 were ascribed to the severe acute pancreatitis (SAP) group. Both SAP and necrotizing AP were associated with higher incidence of MOF, higher mortality and prolonged ICU and overall hospital stay. Whereas mild AP patients showed 0% mortality, overall mortality rate among SAP patients was 27% ($P < 0.01$) and early mortality (during first two weeks from the onset of the disease) was 64% ($P < 0.01$). In the necrotizing AP group overall mortality rate was 25% ($P < 0.01$), and early mortality was 62% ($P < 0.01$), compared to the 1.8% mortality rate seen in the edematous pancreatitis group. The main differences in clinical course and outcome of patients with mild and severe disease are revealed in Table 1. Interestingly, both severe and necrotizing AP were associated with a significant reduction in number of circulating blood lymphocytes, although there was no statistically significant difference in total WBC count (Figure 2). The mean WBC count in the SAP group was 14.76 ± 0.95 × 10^9/L compared to 13.92 ± 0.62 × 10^9/L in the mild AP group (n.s.). The mean WBC count in the necrotizing AP group was 14.38 ± 0.91 × 10^9/L compared to 14.27 ± 0.75 × 10^9/L in the edematous AP group (n.s.). However, the number of circulating lymphocytes in peripheral blood was reduced by 25% in the SAP compared to the mild AP group ($P < 0.05$), and by 17% in the necrotizing AP group compared to the edematous AP group ($P < 0.05$). Based on the significance and clinical relevance of these findings we investigated the presence of possible common molecular pathways leading to development of severe and necrotizing AP.

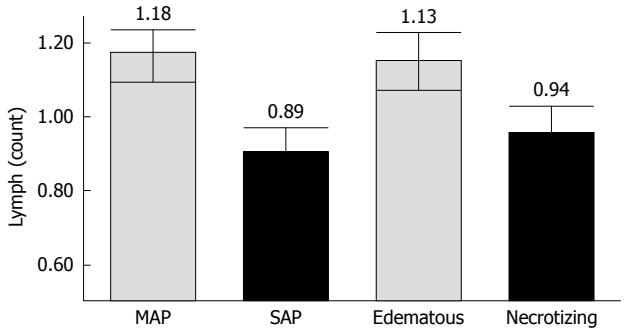


Figure 2 Reduction in number of lymphocytes during severe and necrotizing AP. Number of circulating lymphocytes in peripheral blood was significantly reduced in severe acute pancreatitis (SAP) compared to mild AP groups (0.89 ± 0.08 × 10^9/L *vs* 1.18 ± 0.07 × 10^9/L, $P < 0.05$), and in necrotizing compared to edematous pancreatitis groups (0.94 ± 0.08 × 10^9/L *vs* 1.13 ± 0.08 × 10^9/L, $P < 0.05$). These findings demonstrate the presence of possible common molecular pathways leading to the development of severe and necrotizing AP.

QRT-PCR analysis of cytokine mRNA expression levels in patients with severe and mild disease revealed some conflicting results (Figure 3A). Firstly, study showed that SAP is characterized by decreased intracellular mRNA levels of interleukin (IL)-1 and interferon-γ (IFN-γ). Secondly, SAP was also associated with a marked decrease in the levels of CD25 and CD69 mRNA in peripheral blood leukocytes. However, severe and necrotizing pancreatitis was associated with a very high activation of neutrophils and macrophages as demonstrated by nearly ten-fold higher mRNA expression of elastase and myeloperoxidase in peripheral WBCs. Another quite unexpected result showed a significantly lower mRNA expression of ICAM-1 and macrophage migration inhibitory factor (MIF) in peripheral WBCs (but not in the blood serum) from patients with SAP compared to that of mild AP patients. Analysis of cytokine protein expression levels showed that SAP was associated with a typical SIRS and 2-5 fold increase in expression of pro-inflammatory cytokines (IL-6, IL-8, MIF), as well as induction of compensatory and regulatory mechanism (IL-10) (Figure 3B). Enormously high levels of leukotriene B4 (LTB4) associated with neutrophil activation and injury to the vital organs were recorded in patients who died during the early stages of the disease.

Another important issue is whether the serum soluble molecules could be used as accurate early markers to predict the development of local (pancreatic necrosis) and systemic complications (SIRS, MOF) later in the course of the disease. Discrimination function analysis was used to determine differences between the groups of patients with mild and severe AP, edematous and necrotizing AP, and between survivors and those with fatal outcome. We included five variables (IL-6, IL-8, IL-10, MIF and LTB4) in a study in order to find out which serum markers are the best discriminators of the above mentioned groups. At each step, the variable that minimized the overall Wilks' Lambda was entered into the discriminant model thus revealing that IL-6 and MIF values were significant discriminators between groups of patients with mild and severe disease, as well as between survivors and non-survivors.

Table 2 Serum IL-6 and MIF are significant discriminators between groups of AP patients

Step	Entered	Wilks' Lambda							
		Statistic	df1	df2	df3	Exact F			
						Statistic	df1	df2	*P*
Severe *vs* mild									
1	IL-6	0.739	1	1	70.000	24.696	1	70.000	0.000
2	MIF	0.675	2	1	70.000	16.635	2	69.000	0.000
Edematous *vs* necrotizing									
1	IL-6	0.851	1	1	70.000	12.261	1	70.000	0.001
2	MIF[1]	0.943	1	1	70.000	4.247	1	70.000	0.043
Survivors *vs* non-survivors									
1	IL-6	0.761	1	1	70.000	22.005	1	70.000	0.000
2	MIF	0.709	2	1	70.000	14.153	2	69.000	0.000

[1]Not included in the original model using the standard stepwise method due to its low discrimination value. At each step, the variable that minimizes the overall Wilks' Lambda is entered. IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor.

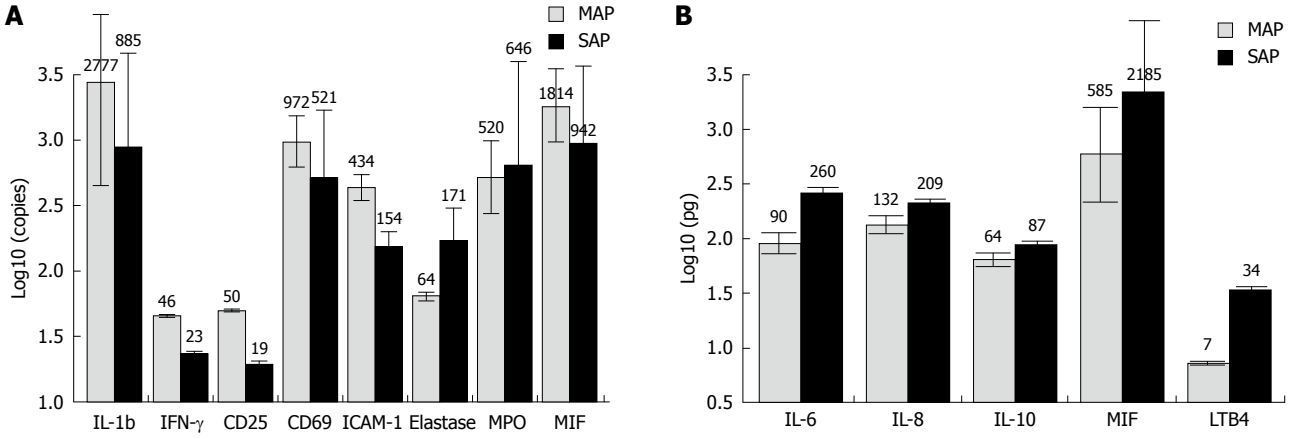
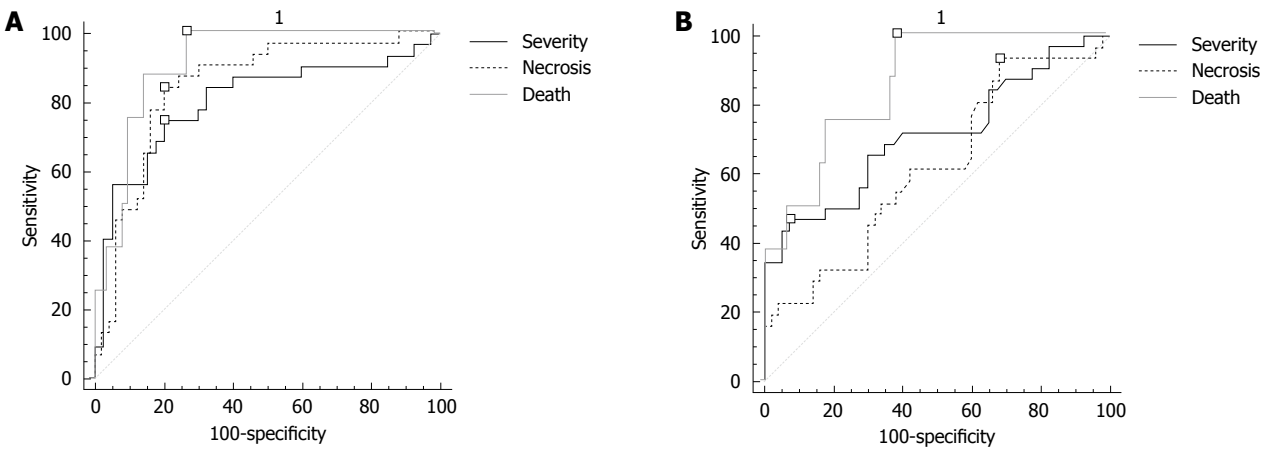


Figure 3 Cytokine expression at mRNA and protein level in peripheral blood of patients with mild and severe AP. A: Gene expression in peripheral blood leukocytes of MAP and SAP patients. QRT-PCR analysis revealed that SAP is characterized by significantly decreased intracellular mRNA levels of interleukin (IL)-1 and interferon (IFN)-γ. SAP is also associated with markedly lower mRNA expression levels of CD25 and CD69 in peripheral blood leukocytes. However, severe and necrotizing pancreatitis was associated with a very high activation of neutrophils and macrophages as demonstrated by nearly ten-fold higher mRNA expression of elastase and myeloperoxidase in peripheral white blood cells (WBCs). A significantly lower mRNA expression of ICAM-1 and macrophage migration inhibitory factor (MIF) was observed in peripheral WBCs (but not in the blood serum) from patients with SAP compared to that of MAP group. Cytokine mRNA expression levels with statistically significant differences between MAP and SAP groups are depicted; B: Cytokine concentration in blood serum of MAP and SAP patients. Analysis of cytokine expression at protein levels shows that SAP is associated with a typical systemic inflammatory response syndrome (SIRS) and 2-5 fold increase in expression of pro-inflammatory cytokines (IL-6, IL-8, MIF). Induction of a compensatory and regulatory mechanism (IL-10) is also observed in this group. Up to 4-5 fold higher levels of LTB4 were recorded in patients who died during the early stages of the disease. Cytokine expression levels with statistically significant differences between AP and healthy control groups are shown.

Statistical analysis revealed that IL-6 values differed significantly in groups of patients with and without pancreatic necrosis (Table 2). A particular cut-off value for IL-6 and MIF was established using Receiver Operating Characteristic (ROC) curve analysis to practically discriminate cases with severe, necrotizing and fatal acute pancreatitis (Figure 4A and B). Comparison of ROC curves for IL-6 and MIF showed no significant differences in diagnostic performance of these two routine tests, except for the discrimination of edematous and necrotizing pancreatitis where IL-6 proved to be of superior value.

DISCUSSION

Alteration of the immune system is one of the major mechanisms responsible for early and late mortality in severe AP. Excessive inflammatory reaction, known as systemic inflammatory response syndrome (SIRS), is considered as the leading cause of death in early AP[14,19,20,29]. The role of lymphocytes in this phenomenon has been partly studied and the knowledge of the mechanisms in humans is still incomplete[20,30-35]. In agreement with previous studies, we found a significant depletion of circulating lymphocytes which was much more profound in the severe and necrotizing forms of pancreatitis[20,30-35]. In our study, significant activation of lymphocytes was observed in the case of AP compared to healthy controls, as shown by strong expression of CD69 and CD25. The surface receptors CD69 and CD25 (IL-2 receptor) are early markers of lymphocyte activation. Increased expression of CD69 as well as of CD25 on CD3+, CD4+ and CD8+ cells have recently been reported in mild AP. It has also been suggested that the number of B-lymphocytes (CD19+) expressing CD69 was significantly lower in patients with



Criterion	Cut-off (pg/mL)	AUC ± SE	*P*	Sens.	Spec.	PPV	NPV
Severity	131.87	0.805 ± 0.05	< 0.01	0.75	0.80	0.74	0.80
Necrosis	83.29	0.790 ± 0.06	< 0.01	0.84	0.73	0.70	0.86
Death	154.82	0.896 ± 0.07	< 0.01	1.00	0.72	0.31	1.00

Criterion	Cut-off (pg/mL)	AUC ± SE	*P*	Sens.	Spec.	PPV	NPV
Severity	1185.74	0.712 ± 0.06	< 0.01	0.47	0.93	0.84	0.69
Necrosis	2706.65	0.550 ± 0.07	0.47	0.23	0.95	0.78	0.62
Death	633.08	0.844 ± 0.09	< 0.01	1.00	0.61	0.24	1.00

Figure 4 Prognostic utility of IL-6 and MIF in diagnosis of severe, necrotizing and fatal AP. A: Performance of serum IL-6 in diagnosis of severe, necrotizing and fatal AP. The diagnostic performance of a test for discriminating severe, necrotizing and fatal cases of AP was evaluated using Receiver Operating Characteristic (ROC) curve analysis. Study revealed that serum IL-6 concentration is a good predictor of the severe disease and systemic complications (SIRS, MOF); this marker could also be utilized for the stratification of patients with necrotizing AP and those with possible fatal outcome; B: Performance of serum MIF in diagnosis of severe, necrotizing and fatal AP. The diagnostic performance of a test for discriminating severe, necrotizing and fatal cases of AP was evaluated using ROC curve analysis. Study revealed that serum MIF concentration is a good predictor of the severe disease and systemic complications (SIRS, MOF); this marker could be used for early identification of patients with possible fatal outcome. Comparison of ROC curves for IL-6 and MIF showed no statistically significant differences in this respect (P = 0.18 for severity; P = 0.58 for mortality). However, serum MIF concentration has very poor prognostic value in predicting the development of pancreatic necrosis and is inferior to IL-6 serum concentration in this respect (P < 0.01). AUC: Area under curve; Sens.: Sensitivity; Spec.: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

severe pancreatitis than in patients with mild pancreatitis[20,31,32]. We have also observed a significantly lower expression of both CD69 and CD25 surface receptors in peripheral blood leukocytes from SAP patients. Marked activation of different subsets of lymphocytes may explain why we have observed strong elevation of different cytokines in peripheral blood, despite a significant decrease in the number of circulating lymphocytes. One of the possible reasons for the decreased number of circulating lymphocytes is strong migration of activated cells to the sites of inflammation, including the pancreas and other tissues such as lungs or kidneys, as an element of SIRS[16,20,36]. Another possible explanation is an excessive elimination of lymphocytes by apoptosis. Interestingly, the available data indicate that lymphopenia caused by the decrease of different lymphocyte subpopulations is also characteristic for other diseases with severe tissue damage, e.g. critical injury, sepsis and acute severe respiratory syndrome (ARDS)[37-40].

Polymorphonuclear leukocytes, monocytes, tissue macrophages, lymphocytes, natural killer cells, and parenchymal cells are involved in a complex network of the host defense response in the case of tissue damage caused by severe AP, burns, accident trauma, major surgical interventions or sepsis[20,39,41,42]. An overwhelming pro-inflammatory response (hyperinflammation) leads to the clinical manifestations of SIRS and finally to host defence failure expressed by multiple organ failure (MOF). The up-regulation of pro-inflammatory factors, such as TNF, IL-1 and IL-6, observed during the SIRS phase can be followed by a second response that involves down-regulation of IFN-γ and increase in anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β. This counter regulatory phenomenon is called the compensatory anti-inflammatory response syndrome (CARS)[17,20,39]. However, this can result in the development of immunosuppression and is often associated with elevation of IL-10 and the shift of the Th1/Th2 balance towards a Th2 response[17,20,39,42]. The network of various cytokines and other molecules participating in the regulation of the inflammatory processes is indeed very complex, and the precise timing of the release and activation of these mediators is not well known. Nevertheless, our study also shows that both SIRS and CARS occur in a real clinical situation and at a certain stage of the disease might even develop simultaneously. However, in the case of severe AP, the counter-regulatory mechanisms may lead to the dysfunction of immune cells mainly due to the significant decrease in total systemic lymphocyte count and the hyper-production of IL-10, which might suggest that T regulatory 1 cells (Tr1) are especially active in the course of severe disease[43].

Involvement of other cellular subsets of leukocytes, mainly neutrophils and monocyte-macrophage lineage, in the development of SAP is also readily supported by our study. Neutrophils exert their toxicity by releasing myeloperoxidase (MPO), proteases such as elastase and reactive oxygen species (ROS) such as H_2O_2[36]. In our study we have observed a very high mRNA expression of MPO and polymorphonuclear leukocyte elastase in cases of severe and necrotizing pancreatitis. Increased production

of platelet activating factor (PAF) and LTB4 is also closely associated with an activation of monocytes and neutrophils, and increased migration of neutrophils to pancreas and other target organs, including lungs and kidneys[36,44,45]. We have also recorded noticeably higher levels of serum LTB4 in patients suffering from severe and fatal acute pancreatitis. Furthermore, patients with severe and necrotizing AP exerted very high serum levels of MIF. MIF is predominantly released by monocytes, macrophages and Th2 T-cell clones; it is associated with activation of immune cells and release of pro-inflammatory cytokines overriding glucocorticoid action at the site of inflammation. It is also known to be associated with a lung injury during SIRS through counter-regulation of the effect of glucocorticoids and through up-regulation of macrophage inflammatory protein-2, which itself serves as a potent chemo-attractant to the neutrophils[46-49].

The infiltration of inflammatory cells into the pancreas is an early and central event in acute pancreatitis that promotes local injury and systemic complications of the disease. Recent research has yielded the important finding that resident cells of the pancreas (particularly acinar and pancreatic stellate cells) play an important role in leukocyte attraction *via* secretion of chemokines, cytokines and expression of adhesion molecules[36,50-53]. We believe this could at least partially explain the occurrence of systemic lymphopenia, lower CD25, CD69 and ICAM-1 mRNA expression in cases of SAP. Activation of inflammatory cells and release of cytokines into the systemic bloodstream at the site of injury is possibly also an explanation for the discrepancy of low MIF mRNA expression in peripheral WBCs in association with high serum concentration of MIF in the case of severe and necrotizing pancreatitis.

Having identified the inflammatory mediators that are significantly up-regulated in serum of patients with severe and necrotizing AP (IL-6, IL-8, IL-10, MIF and LTB4) in comparison to those with mild disease, we have also evaluated the prognostic utility of these molecules in predicting the negative outcome of the disease. In accordance with the findings of other studies we have confirmed that both serum IL-6 and MIF concentrations are very good discriminators of severe AP accompanied by MOF, and could be used as early markers of possible fatal outcome. However, in contrast to the recent study of Rahman *et al*[54], we failed to demonstrate that MIF is an early specific prognostic marker for the development of pancreatic necrosis. In our experience, serum IL-6 concentration was superior in this respect[28,55-57]. Nonetheless, serum MIF concentration may serve as an important prognostic marker that could identify patients likely to benefit from aggressive medical treatment and early transfer to specialist pancreatic units. At the molecular level, targeted disruption of these proteins with monoclonal antibodies may afford some therapeutic benefit in preventing the development of MOF, pancreatic necrosis, and septic complications.

Deregulation of the cellular immune system is a key event leading to the development of severe and necrotizing AP. Severe and necrotizing AP is characterized by the significant depletion of circulating lymphocytes in the bloodstream as a result of increased migration of these cells to the site of injury and other vital organs, including lungs and kidneys. Infiltration of pancreas and other peripheral organs by aberrantly activated inflammatory cells occurs within the first hours after onset of acute pancreatitis and leads to the development of SIRS and MOF. This whole process is regulated by a large number of cytokines and is an extremely complex network of intercellular interaction. It is clear that detailed elucidation of the numerous processes in the inflammatory cascade is an essential step towards the development of improved therapeutic strategies in acute pancreatitis. Published studies suggest that combination therapy targeting different steps of the inflammatory cascade may be the treatment of choice for this disease.

Data from other authors and from our current study demonstrate that serum IL-6 and MIF concentrations are very good discriminators of severe and necrotizing AP as well as of possible fatal outcome during the first hours and days after the onset of the disease. Therefore, these molecules could be used as markers for patient stratification and/or predicting the outcomes of AP in the early stages of the disease. However, their true prognostic utility, clinical and health care resource implications need to be further evaluated in a larger patient population and in the context of other currently existing diagnostic markers and indices.

COMMENTS

Background

Mortality of patients with severe acute pancreatitis (SAP) approaches 30%-40%. An imbalance between the early systemic inflammatory response syndrome (SIRS), and the later compensatory counter-inflammatory response, and development of multiple organ failure (MOF) are considered to be the primary causes of morbidity and mortality in SAP. Excessive leukocyte activation (including neutrophils and monocyte-macrophage lineage) with cytokinemia play a critical role in the pathogenesis of pancreatitis and even more so, of the subsequent inflammatory response. Thus, the aims of this study have been to investigate the secretion patterns of pro- and anti-inflammatory cytokines, and to estimate the activation of various cellular subsets of leukocytes (including lymphocytes, neutrophils, and monocyte-macrophage lineage) in peripheral blood of patients with severe and mild AP.

Research frontiers

It has been hypothesized that fatal pancreatitis is a consequence of abnormal phagocytic leukocyte hyperstimulation due to deregulation in T- and B-lymphocyte activation. However, the role of lymphocyte activation and its relation to the severity of disease in humans is still poorly understood. Another important drawback is that the majority of information about the alterations of the immune system during the AP comes from *in vitro* and *in vivo* studies, and therefore is not always directly applicable and relevant to the clinical situation in human AP.

Innovations and breakthroughs

This study by Dambrauskas *et al* confirms that human severe and necrotizing AP is characterized by the significant depletion of circulating lymphocytes. SAP is associated with a severe systemic inflammatory response syndrome (SIRS) and over-expression of pro-inflammatory cytokines [interleukin (IL)-6, IL-8, macrophage migration inhibitory factor (MIF)]. The study demonstrates that serum IL-6 and MIF concentrations are the best discriminators of severe and necrotizing AP as well as possible fatal outcome during the early course of the disease. These cytokines could also be used as early predictors of local (pancreatic necrosis) and systemic complications (SIRS, MOF) later in the course of the disease. Deregulation of the cellular immune system is a key event leading to

the development of severe and necrotizing AP. Infiltration of peripheral organs by aberrantly activated inflammatory cells leads to the development of MOF.

Applications

Data from other authors and from the authors' current study demonstrate that serum IL-6 and MIF concentrations are very good discriminators of severe and necrotizing AP as well as possible fatal outcome during the first hours and days after the onset of the disease. Therefore, these molecules could be used as markers for patient stratification and/or predicting the outcome of AP in the early stages of the disease.

Terminology

Pancreatitis is a condition associated with development of acute and sudden inflammation of the pancreas. In about 85% of patients, acute pancreatitis is a mild disease and is usually associated with a rapid recovery within a few days of onset of the illness. However, in 15%-20% of patients with acute pancreatitis, severe damage to the pancreas may lead to a life threatening illness that is often associated with prolonged hospitalization, multiple surgical procedures, development of multiple organ failure, severe infective complications, and death in some patients.

Peer review

This prospective observational study seeks to characterise the cytokine expression profiles (at mRNA and protein levels) of patients with acute pancreatitis and to correlate the profiles with disease severity.

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ORIGINAL ARTICLE

Insulin-like growth factor-I receptor in proliferation and motility of pancreatic cancer

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Abstract

AIM: To develop a molecular therapy for pancreatic cancer, the insulin-like growth factor-I (IGF-I) signaling pathway was analyzed.

METHODS: Pancreatic cancer cell lines (MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4) were cultured in media with 10 mL/L fetal bovine serum. Western blotting analysis was performed to clarify the expression of IGF-I receptor (IGF-IR). Picropodophyllin (PPP), a specific inhibitor of IGF-IR, LY294002, a specific inhibitor of phosphatidylinositol 3 kinase (PI3K), and PD98059, a specific inhibitor of mitogen-activated protein kinase, were added to the media. After 72 h, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed to analyze cell proliferation. A wound assay was performed to analyze cell motility with hematoxylin and eosin (HE) staining 48 h after addition of each inhibitor.

RESULTS: All cell lines clearly expressed not only IGF-IR but also phosphorylated IGF-IR. PPP significantly suppressed proliferation of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to 36.9% ± 2.4% (mean ± SD), 30.9% ± 5.5%, 23.8% ± 3.9%, 37.1% ± 5.3%, 10.4% ± 4.5%, 52.5% ± 4.5% and 22.6% ± 0.4%, at 2 μmol/L, respectively ($P < 0.05$). LY294002 significantly suppressed proliferation of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to 44.4% ± 7.6%, 32.9% ± 8.2%, 53.9% ± 8.0%, 52.8% ± 4.0%, 32.3% ± 4.2%, 51.8% ± 4.5%, and 30.6% ± 9.4%, at 50 μmol/L, respectively ($P < 0.05$). PD98059 did not significantly suppress cell proliferation. PPP at 2 μmol/L suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to 3.0% ± 0.2%, 0%, 0%, 2.0% ± 0.1%, 5.0% ± 0.2%, 3.0% ± 0.1%, and 5.0% ± 0.2%, respectively ($P < 0.05$). LY294002 at 50 μmol/L suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 to 3.0% ± 0.2%, 0%, 3.0% ± 0.2%, 0%, 0%, 0% and 3% ± 0.1%, respectively ($P < 0.05$). PD980509 at 20 μmol/L did not suppress motility. Cells were observed by microscopy to analyze the morphological changes induced by the inhibitors. Cells in medium treated with 2 μmol/L PPP or 50 μmol/L LY294002 had pyknotic nuclei, whereas those in medium with 20 μmol/L PD98059 did not show apoptosis.

CONCLUSION: IGF-IR and PI3K are good candidates for molecular therapy of pancreatic cancer.

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Key words: Insulin-like growth factor-I receptor; Phosphatidylinositol 3 kinase; Pancreatic neoplasms

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INTRODUCTION

Molecular alterations of pancreatic cancer need to be clarified to develop molecular therapy. Growth factors transmit signals to stimulate tumor growth and enhance metastasis. Insulin-like growth factor (IGF)-I signaling plays an important role in the growth and development of many tissues[1]. IGF-I signaling is also thought to be involved in tumorigenesis. Upon ligand binding, the tyrosine kinase of IGF-I receptor (IGF-IR) is activated, and results in autophosphorylation of tyrosine[2]. This initiates a phosphorylation cascade to the phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways.

IGF-I is upregulated in human pancreatic cancer tissues but is not expressed in surrounding non-cancerous tissue[3]. Serum level of IGF-I is elevated in pancreatic cancer patients[4]. Histological analysis has shown that IGF-IR is positive in the membrane of cells of pancreatic cancer tissues but is not expressed in surrounding non-tumorous tissues[3,5]. These facts imply that IGF-I acts as a growth factor for pancreatic cancer. IGF-IR is phosphorylated solely in pancreatic cancer tissues, and not in surrounding non-tumorous tissues[6]. The present study suggests that IGF-IR transmits signals to downstream pathways. This hypothesis is supported by the facts that IGF-I stimulates DNA synthesis of PANC-1 and MIA Paca2 pancreatic cancer cell lines, and motility of HPAF-II, another pancreatic cancer cell line[7,8].

IGF-IR is not involved in metabolism, therefore, it was expected that inhibition of IGF-IR and its downstream pathway would not cause any adverse effects[1]. We therefore focused on the IGF-IR signaling pathway. Pancreatic cancer tissues express growth factors other than IGF-I[9]. We used fetal bovine serum (FBS) as a growth model of pancreatic cancer cells instead of addition of IGF-I in serum-free medium, because pancreatic cancer tissues and serum from patients contain various growth factors.

MATERIALS AND METHODS

Culture and reagents

Pancreatic cancer cell lines, PANC-1, NOR-P1, PK-45H, PK-1, PK-59, and MIA-Paca2 were purchased from RIKEN Cell Bank (Tsukuba, Japan)[10]. NOR-P1 was cultured in Dulbecco's Minimum Essential Medium (DMEM): F12 medium and MIA-Paca2 was cultured in DMEM, and the others in Roswell Park Memorial Institute (RPMI)-1640 (Sigma, St. Louis, MO, USA). All the media were purchased from Sigma, and supplemented with 100 mL/L FBS (Trace Scientific, Melbourne, Australia). All the cell lines were cultured in 50 mL/L carbon dioxide at 37℃ in a humidified chamber. LY294002, a specific inhibitor of PI3K, and PD98059, a specific inhibitor of MAPK, were purchased from Wako Pure Chemicals (Osaka, Japan), and picropodophyllin (PPP), a specific inhibitor of IGF-IR, was from Calbiochem (Darmstadt, Germany)[11].

Western blotting analysis

Protein was isolated from cells after 72 h culture. Twenty micrograms of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nylon filter. Primary antibodies were polyclonal rabbit anti-IGF-IR antibody, anti-phosphorylated IGF-IR (Cell Signaling Technology, Danvers, MA, USA), and mouse monoclonal anti-tubulin-α antibody (Lab Vision, Fremont, CA, USA). Second antibodies were horseradish peroxidase (HRP)-linked anti-rabbit antibody (Amersham Bioscience, Tokyo, Japan) and HRP-linked anti-mouse antibody (Amersham Bioscience). Dilutions were 1:500 for primary antibodies, and 1:1000 for second antibodies. The filter was reprobed with anti-tubulin-α antibody. The specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA).

Cell proliferation assay

Cells were trypsinized, harvested, and spread onto 96-well flat-bottom plates (Asahi Techno Glass, Tokyo, Japan) at a density of 1000 cells per well. Following 24 h of culture under RPMI-1640, DMEM, or DMEM: F12 with 100 mL/L FBS, medium was changed to RPMI-1640, DMEM, or DMEM: F12 without FBS, respectively, to quench the FBS effects. After 24 h of culture, the medium was changed to RPMI-1640, DMEM, or DMEM:F12 with 100 mL/L FBS, in addition to LY294002, PD98059, or PPP. After 72 h, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed according to the manufacturer's instructions (Promega Corporation, Tokyo, Japan). MTS was bioreduced by cells into a colored formazan product that reduced absorbance at 490 nm. Absorbance was analyzed with a multiple plate reader at a wavelength of 490 nm with a BIO-RAD iMark microplate reader (Bio-RAD, Hercules, CA, USA).

Wound assay

Wound assays were performed according to a previously described procedure[12]. Briefly, cells were spread onto four-well chambers (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were then cut with a sterile razor, and stained with hematoxylin and eosin (HE). Five images were taken by a light microscope (Olympus, Tokyo, Japan). For each

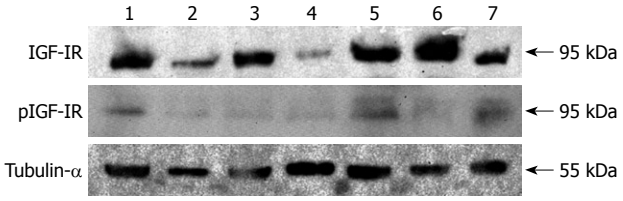

Figure 1 Western blotting analysis of pancreatic cell lines. Protein was isolated from cultured pancreatic cancer cell lines in 100 mL/L fetal bovine serum (FBS), and subjected to western blot analysis. It was shown clearly that all cell lines expressed IGF-IR as well as phosphorylated IGF-IR (pIGF-IR). Tubulin-α was used as an internal control. 1: MIA-Paca2; 2: NOR-P1; 3: PANC-1; 4: PK-45H; 5: PK-1; 6: PK-59; 7: KP-4.

experiment, the number of cells that migrated more than 150 μm per 100 μm cut surface was counted.

Statistical analysis

Cell proliferation and wound assay were analyzed statistically by one-factor analysis of variance. Statistical analysis was performed with JMP5.0J (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was accepted as statistically significant.

RESULTS

Expression and phosphorylation of IGF-IR in pancreatic cancer

To reveal involvement of IGF-IR in proliferation of pancreatic cancer cell lines stimulated with FBS, protein was isolated and subjected to Western blotting analysis. All cell lines clearly expressed not only IGF-IR but also phosphorylated IGF-IR, which suggested that IGF-IR played a role in proliferation with FBS (Figure 1). This result prompted us to analyze the effects of IGF-IR inhibitors.

Suppression of proliferation of pancreatic cancer with inhibitors

The MTS assay was performed to clarify whether PPP, LY294002, or PD98059 suppressed proliferation of pancreatic cancer cell lines. PPP suppressed proliferation of all the cell lines examined (Figure 2A). At 2 μmol/L, MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 proliferation was reduced to 36.9% ± 2.4% (mean ± SD), 30.9% ± 5.5%, 23.8% ± 3.9%, 37.1% ± 5.3%, 10.4% ± 4.5%, 52.5% ± 4.5% and 22.6% ± 0.4%, respectively ($P < 0.05$, $n = 3$). Next, we analyzed the downstream pathway of IGF-IR. LY294002 suppressed proliferation of all the cell lines examined (Figure 2B). At 50 μmol/L, proliferation of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells was reduced significantly to 44.4% ± 7.6%, 32.9% ± 8.2%, 53.9% ± 8.0%, 52.8% ± 4.0%, 32.3% ± 4.2%, 51.8% ± 4.5%, and 30.6% ± 9.4%, respectively ($P < 0.05$, $n = 3$). PD98059 did not suppress cell proliferation (Figure 2C). Although NOR-P1 and KP-4 cells showed a marginal decrease in proliferation in the presence of 20 μmol/L PD98059, we could not analyze higher concentrations because PD98059 precipitated in the medium. Analysis with 50 μmol/L PD98059 failed since PD98059 crystallized and precipitated. The other inhibitor of MAPK was not analyzed.

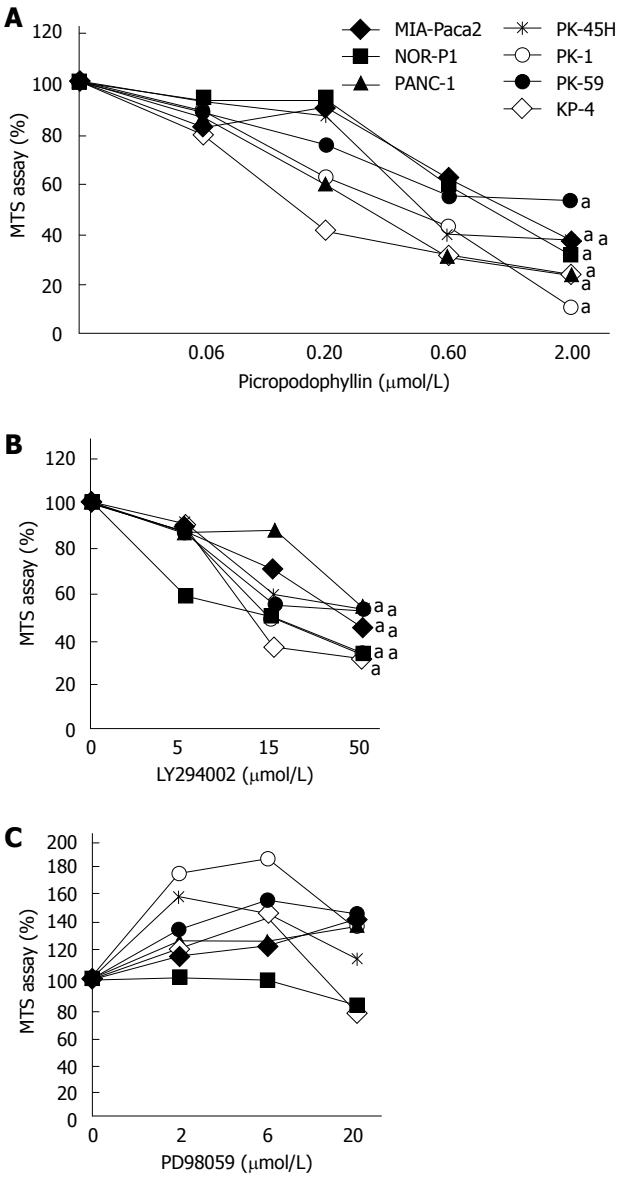

Figure 2 MTS assay of cells cultured with inhibitors. Pancreatic cell lines were cultured in 100 mL/L FBS with picropodophyllin (PPP) (A), LY294002 (B), or PD98059 (C). After 72 h, MTS assay was performed to analyze the change in cell numbers. PPP and LY294002 significantly suppressed cell numbers, whereas PD98059 did not ($^aP < 0.05$, $n = 3$).

Suppression of motility of pancreatic cancer with inhibitors

Suppression of motility is the initial step in the inhibition of metastasis. We analyzed changes in motility with inhibitors, by the wound assay (Figure 3). PPP at 2 μmol/L suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to 3.0% ± 0.2%, 0%, 0%, 2.0% ± 0.1%, 5.0% ± 0.2%, 3.0% ± 0.1%, and 5.0% ± 0.2%, respectively ($P < 0.05$, $n = 3$). LY294002 at 50 μmol/L suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to 3.0% ± 0.2%, 0%, 3.0% ± 0.2%, 0%, 0%, 0% and 3% ± 0.1%, respectively ($P < 0.05$, $n = 3$). PD980509 at 20 μmol/L did not suppress motility, although NOR-P1 cells showed a marginal, non-significant decrease.

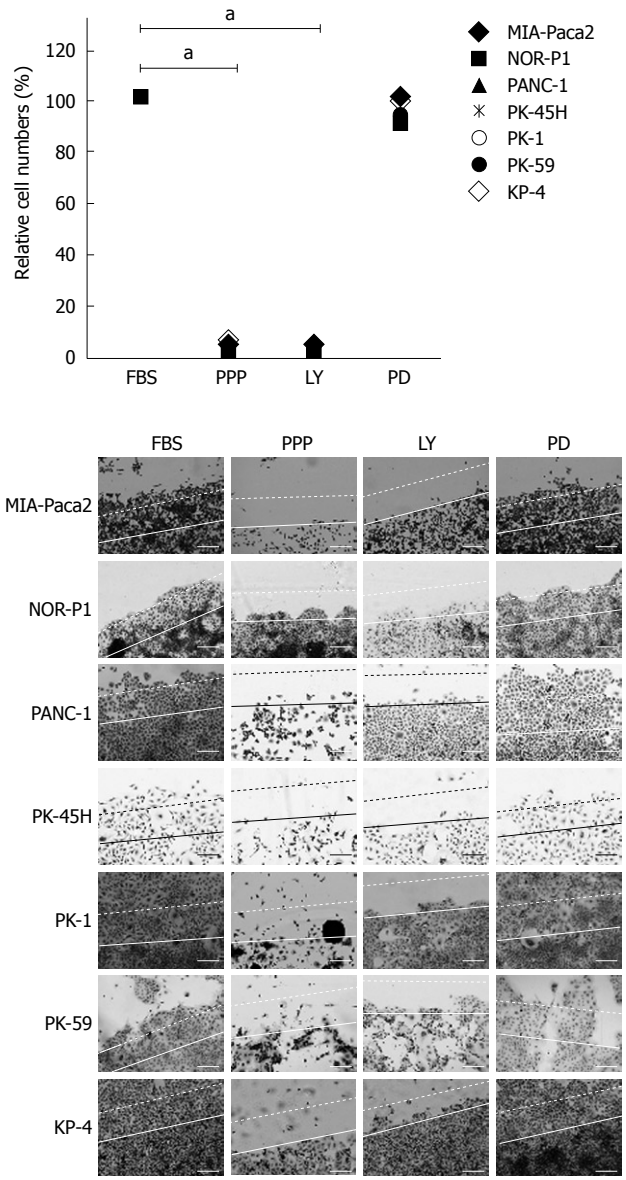

Figure 3 Wound assays with inhibitors. Wound assay was performed to analyze cell motility. Almost no cells cultured with PPP or LY294002 migrated > 150 μm, whereas those with PD98059 showed motility of > 150 μm ($^aP < 0.05$, $n = 3$). Solid line: Edge of scratch; Dotted line: 150 μm from a solid line. Original magnification: 4 ×; Scale bar: 100 μm. FBS: Medium with 100 mL/L FBS without any inhibitors; PPP: Medium with 2 μmol/L PPP; LY: Medium with 50 μmol/L LY294002; PD: Medium with 20 μmol/L PD98059.

Microscopic features of pancreatic cancer with inhibitors

Cells were observed under the microscope to analyze the morphological changes induced by inhibitors. Cells in medium with 2 μmol/L PPP or 50 μmol/L LY294002 had pyknotic nuclei, which suggested that they were apoptotic (Figure 4). Cells in medium with 20 μmol/L PD98059 did not show signs of apoptosis (Figure 4).

DISCUSSION

IGF-I stimulates thymidine incorporation of pancreatic cancer cell lines, such as MIA-Paca2, as strongly as FBS, which suggests that it is an autocrine growth factor[13]. It

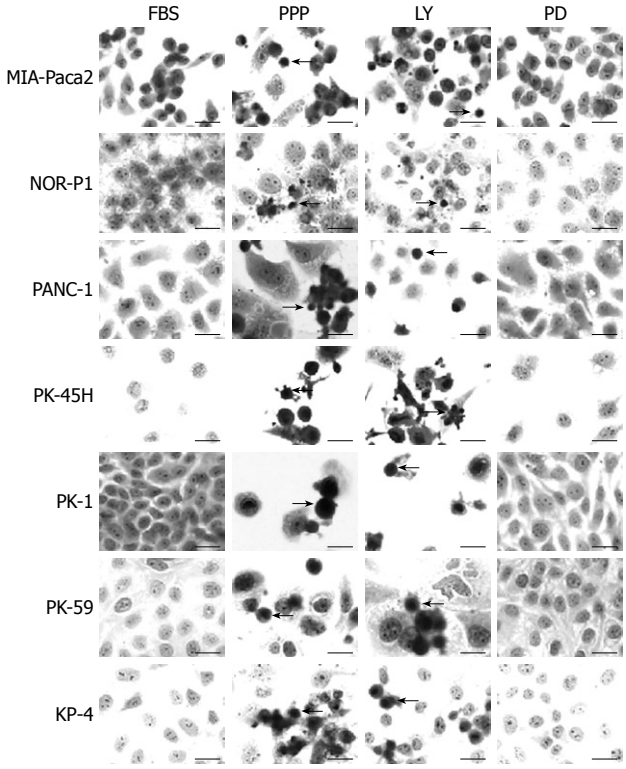

Figure 4 Apoptotic cells with PPP or LY294002. Under the microscope, cells with pyknotic nuclei (arrows) were observed in medium with PPP or LY294002, whereas there were no apoptotic cells in medium with PD98059. FBS: Medium with 100 mL/L FBS; PPP: Medium with 2 μmol/L PPP; LY: Medium with 50 μmol/L LY294002; and PD: Medium with 20 μmol/L PD98059. Original magnification: 20 ×; Scale bar: 25 μm, the arrows indicate cells in apoptosis.

was expected that inhibition of IGF-IR activity would lead to pancreatic cancer regression. Antisense oligonucleotide to IGF-IR suppresses proliferation of ASPC-1, a pancreatic cancer cell line[3]. Antisense oligonucleotide to K-ras synergistically enhances the suppression of pancreatic cancer cell lines by antisense oligonucleotide to IGF-IR[14]. Antisense oligonucleotide is unstable in blood, therefore, small molecules that inhibit the tyrosine kinase of IGF-IR are desirable. NVP-AEW541, an IGF-IR inhibitor, suppresses growth of HPAF-II cells, a pancreatic cancer cell line[15]. However, it is possible that NVP-AEW541 does not affect other cell lines. PPP is the first inhibitor to discriminate IGF-IR and insulin receptor[16]. In our experiments, PPP suppressed proliferation of all pancreatic cancer cell lines. Previous studies and the present one indicate that IGF-IR is a good candidate for molecular therapy of pancreatic cancer.

The downstream pathway of the IGF-I receptor is intriguing since shutting down the pathway could reduce cancer. Antibody to IGF-IR suppresses phosphorylation of Akt, a downstream molecule of PI3K and MAPK[13]. LY294002 significantly suppresses proliferation of PANC-1 and BxPC3 cells, and viability of KP-4 and PANC-1 cells[7,17]. Our results showed that LY294002 suppressed proliferation of all the pancreatic cancer cell lines examined. In addition to proliferation, our data clearly demonstrated that LY294002 suppressed cell migration. We conclude that PI3K is a suitable target for molecular therapy of pancreatic cancer.

MAPK is another downstream pathway of IGF-IR. MIA-PaCa2 treated with 20 μmol/L PD98059 in 100 mL/L FBS showed downregulation of phosphorylated extracellular signal-regulated kinase 1/2[18]. Although it was expected that PD98059 would suppress proliferation of pancreatic cancer cells, 20 μmol/L PD98059 did not suppress proliferation and motility of pancreatic cancer cells in our experiments. PD98059 at 20 μmol/L does not suppress DNA synthesis stimulated by IGF-I in the culture medium, although phosphorylation of MAPK is suppressed[19]. Curiously, PD98059 stimulates cell growth at concentrations of 0.1 μmol/L to 0.1 pmol/L[20]. SB203580, another inhibitor of MAPK, stimulates growth of PANC-1 cells in serum-free conditions[21]. In our experiments, PD98059 marginally stimulated growth of PANC-1 cells with 10 mL/L FBS. These results suggest that inhibition of MAPK does not always suppress growth of pancreatic cancer cell lines with unknown mechanisms.

It may be concluded that IGF-IR and PI3K are good candidates for molecular therapy of pancreatic cancer.

COMMENTS

Background

Insulin-like growth factor-I receptor (IGF-IR) is expressed in pancreatic cancer tissues but not in surrounding non-cancerous tissues. Although it was speculated that IGF-I might play a role in proliferation of pancreatic cancer, the detailed mechanism is not known.

Research frontiers

IGF-IR is not involved in metabolism, therefore, it is expected that inhibition of IGF-IR and its downstream pathway should not cause adverse effects when used as a target for molecular therapy of pancreatic cancer.

Innovations and breakthroughs

IGF-IR was phosphorylated in cultured pancreatic cancer cell lines, which suggested that IGF-IR and its downstream pathway were activated. Each inhibitor of IGF-IR or phosphatidylinositol-3 kinase (PI3K) suppressed not only cell proliferation but also motility.

Applications

By revealing that IGF-IR was activated, this study indicated that IGF-IR might be a good candidate for molecular therapy of pancreatic cancer.

Terminology

IGF-I is a growth factor that is involved in cell proliferation and differentiation. Stimulation of IGF-I is transmitted *via* IGF-IR to downstream pathways, such as PI3K and mitogen-activated protein kinase.

Peer review

The authors have demonstrated that IGF-IR, phosphorylated and total, is involved in the proliferation of pancreatic cell lines. In Figure 1, we see that all cell lines expressed phosphorylated and total IGF-IR. Their experiments have shown that inhibition of IGF-IR activity results in a decrease in proliferation and motility of pancreatic cancer cell lines. These findings, along with the inhibition of PI3K, are interesting and show promise.

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ORIGINAL ARTICLE

Effect of S1P5 on proliferation and migration of human esophageal cancer cells

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Abstract

AIM: To investigate the sphingosine 1-phosphate (S1P) receptor expression profile in human esophageal cancer cells and the effects of S1P5 on proliferation and migration of human esophageal cancer cells.

METHODS: S1P receptor expression profile in human esophageal squamous cell carcinoma cell line Eca109 was detected by semi-quantitative reverse transcription polymerase chain reaction. Eca109 cells were stably transfected with S1P5-EGFP or control-EGFP constructs. The relation between the responses of cell proliferation and migration to S1P and S1P5 expression was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and migration assay, respectively.

RESULTS: Both normal human esophageal mucosal epithelium and Eca109 cells expressed S1P1, S1P2, S1P3 and S1P5, respectively. Esophageal mucosal epithelium expressed S1P5 at a higher level than Eca109 cell line. S1P5 over-expressing Eca109 cells displayed spindle cell morphology with elongated and extended filopodia-like projections. The proliferation response of S1P5-transfected Eca109 cells was lower than that of control vector-transfected cells with or without S1P stimulation ($P < 0.05$ or 0.01). S1P significantly inhibited the migration of S1P5-transfected Eca109 cells ($P < 0.001$). However, without S1P in transwell lower chamber, the number of migrated S1P5-transfected Eca109 cells was greater than that of control vector-transfected Eca109 cells ($P < 0.001$).

CONCLUSION: S1P binding to S1P5 inhibits the proliferation and migration of S1P5-transfected Eca109 cells. Esophageal cancer cells may down-regulate the expression of S1P5 to escape the inhibitory effect.

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Key words: Sphingosine 1-phosphate; Esophageal cancer; Sphingosine 1-phosphate 5; Proliferation; Migration

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INTRODUCTION

Esophageal cancer is one of the most common malignancies worldwide, occurring mostly in developing countries with marked regional variations in incidence. Its mortality is very similar to its incidence due to relatively late diagnosis and inefficient treatment. About 300 000 people would die of esophageal cancer each year in the world[1]. The incidence and mortality of esophageal cancer are unusually high in China, especially in Henan, Shanxi, Hebei and Sichuan Provinces[2]. Even though a small number of esophageal cancer patients can survive more than 5 years after initial surgical treatment, over 60% of patients still die of metastasis and local recurrence[3,4]. The ability to reverse the outcome of esophageal cancer is limited due to a poor understanding of its biology.

Sphingosine 1-phosphate (S1P) is a pleiotropic phospholipid mediator that mediates diverse cellular responses, such as cell proliferation, differentiation, migration, adhesion, and morphogenesis. S1P with a low nanomolar affinity binds to its 5-related G-protein coupled receptors (GPCR), and is designated as S1P1-5 (originally termed EDG-1, 3, 5, 6, and 8, respectively). S1P1, S1P2, and S1P3 receptors are ubiquitously expressed, whereas S1P4 is only expressed in lymphoid tissues and platelets, and S1P5 is predominantly expressed in the central nervous system[5-11].

Previous reports have highlighted the importance of S1P and its receptors for the growth, metastasis and infiltration of tumors, such as gastric cancer[5], thyroid cancer[12], ovarian cancer[13-16], and glioma[17]. In general, S1P stimulates the migration of S1P3 dominantly expressing cell lines and inhibits the migration of S1P2 expressing cell lines. S1P1, S1P2 and S1P3 contribute positively to S1P-stimulated glioma cell proliferation, and S1P5 blocks glioma cell proliferation. S1P1 and S1P3 enhance glioma cell migration and invasion, while S1P2 inhibits cell migration but unexpectedly enhances glioma cell invasiveness by stimulating cell adhesion[17].

The possible role and importance of S1P and its receptors in the growth and metastasis of human esophageal cancer have not been investigated. In this study, we focused on esophageal squamous cell carcinoma (ESCC), characterized S1P receptor expression patterns and investigated the role of S1P receptors in proliferation and migration of ESCC cells. ESCC cell line Eca109 expressed 4 S1P receptors (S1P1, S1P2, S1P3 and S1P5). Compared with normal esophageal mucosa epithelium, Eca109 cells expressed a very low level of S1P5. Furthermore, by overexpressing human S1P5 in Eca109 cells, the effects of S1P5 on esophageal cancer cells were partly clarified.

MATERIALS AND METHODS

Materials

S1P was obtained from Cayman Chemical (Ann Arbor, MI, USA). Dispase Ⅱ, fibronectin and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). SV total RNA isolation system and pGEM-T easy vector system were bought from Promega (Madison, WI, USA). PrimeScript™ reverse transcription polymerase chain reaction (RT-PCR) kit and PrimerSTAR HS DNA polymerase with GC buffer were purchased from TakaRa Biotech (Dalian, China). Lipofectamine 2000 and G418 were obtained from Invitrogen (Carlsbad, CA, USA).

Preparation of normal human esophageal mucosal epithelium

Normal human esophageal mucosa samples were obtained from surgically resected esophagus of patients with esophageal carcinoma, and then confirmed by an experienced pathologist to contain neither macroscopic tumor nor histologically detectable metaplastic cells or cancer cells. Samples were collected with the signed informed consent from patients. Tunica mucosa was separated from the tissues with forceps, and cut into 1 cm × 1 cm sections which were then transferred to a 100 mm culture dish containing 10 mL PBS with 0.25% dispase Ⅱ and incubated for 18-24 h at 4℃. Epithelial layers were separated from the full-thickness mucosa with forceps.

Cell culture

Human ESCC cell line Eca109 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Eca109 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 U/mL of penicillin and streptomycin at 37℃ in a humidified incubator containing 5% CO_2.

Preparation of total RNA and semi-quantitative reverse transcription polymerase chain reaction

Total RNA was isolated with the SV total RNA isolation system following its manufacturer's instructions. RNA quality and integrity were checked by absorbance spectrometry and agarose gel electrophoresis. Semi-quantitative RT-PCR was performed to confirm differential expression of S1P receptors in Eca109 cell line. The sequences of primers used are as follows: 5'-TATCAGCGCGGACAAGGAGAACAG-3' (sense) and 5'-ATAGGCAGGCCACCCAGGATGAG-3' (antisense) for S1P1 receptor (429-bp product), 5'-TCGGCCTTCATCGTCATCCTCT-3' (sense) and 5'-CCTCCCGGGCAAACCACTG-3' (antisense) for S1P2 receptor (220-bp product), 5'-CTGCCTGCACAATCTCCCTGACTG-3' (sense) and 5'-GGCCCGCCGCATCTCCT-3' (antisense) for S1P3 receptor (394-bp product), 5'-GAGAGCGGGGCCACCAAGAC-3' (sense) and 5'-GGTTGACCGCCGAGTTGAGGAC-3' (antisense) for S1P4 receptor (454-bp product), 5'-GGAGTAGTTCCCGAAGGACC-3' (sense) and 5'-TCTAGAATCCACGGGGTCTG-3' (antisense) for S1P5 receptor (236-bp product), 5'-GATGACATCAAGAAGGTGGTGAA-3' (sense) and 5'-GTCTTACTCCTTGGAGGCCATGT-3' (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (246-bp product). Thirty-two cycles of amplification were performed using a thermal

cycler, Mastercycle® gradient (Eppendorf, Hamburg, Germany) at 94℃ for 30 s, at 60℃ for 30 s, and at 72℃ for 1 min. The PCR products were electrophoresed on a 1.6% agarose gel and visualized with ethidium bromide staining.

Generation of stably-transfected Eca109 cell line

Human S1P5 open reading frame was cloned from normal esophageal mucosal epithelium cDNA *via* PCR using the PrimerSTAR HS DNA polymerase with GC buffer. The sequences of primers used are 5'-CATTG AAGCTTCCACC*ATG*GAGTCGGGGCTGCTG-3' (sense) and 5'-CATTGTCTAGAGTCTGCAGCCG GTTCTGATA-3' (antisense). The sense primer was designed to add a *Hin*dⅢ site upstream of a consensus Kozak sequence and the S1P5 initiating Met (italics). The antisense primer was designed to remove the S1P5 stop codon and to add a *Xba* I site. PCR conditions, established to amplify the S1P5 receptor sequence, were 94℃ for 1 min, followed by 35 cycles at 94℃ for 30 s, at 60℃ for 30 s, at 72℃ for 2 min, and a final extension at 72℃ for 8 min. The PCR products were electrophoresed on 0.8% agarose gel and the corresponding band was cut and purified. The purified PCR products were ligated into the pGEM-T easy vector and the sequence was confirmed by sequence analysis. The *S1P5* gene was spliced out with *Hin*dⅢ/*Xba* I and ligated into the Myc-His-EGFP-N1 frame, which was purified from similarly digested S1P1-Myc-His-EGFP-N1 as previously described[18]. The initiating methionine of the GFP open reading frame in pEGFP-N1 was mutated to alanine. The resulting plasmid was designated as S1P5-EGFP. A *Hin*dⅢ/*Xba* I -digested DNA oligonucleotide containing both sense (5'-CATTGAAGCTTCCACCATGGAATTCTGCAGT CGACGGTACTCTAGACCG-3') and antisense (5'-CG GTCTAGAGTACCGTCGACTGCAGAATTCCATG GTGGAAGCTTCAATG-3') sequences was cloned into similarly digested Myc-His-EGFP-N1 frame as a control vector. The DNA oligonucleotide contained initiating Met and multiple cloning sites. The plasmid was designated as control-EGFP.

Cells were transfected using Lipofectamine 2000, according to its manufacturer's instructions. Briefly, 5×10^5 Eca109 cells in 2 mL of growth medium without antibiotics were seeded into each well of 6-well plates and grown overnight, then transfected using Lipofectamine 2000. The cells were split at a 1:20 dilution into a fresh growth medium 24 h after transfection. G418 was added at a concentration of 0.8 mg/mL the following day. Resistant colonies were picked to a 96-well plate 14 d after selection and several clones were analyzed. The cells were subsequently maintained in a medium containing G418 at 0.2 mg/mL.

Proliferation assay

Cells (5×10^3/well in 100 μL/well) were seeded into a 96-well plate and cultured in RPMI 1640 medium containing 10% FBS for 8 h at 37℃ in an atmosphere containing 5% CO_2 to allow attachment. The medium was then aspirated and a fresh medium containing 0.1% fatty acid-free BSA and varying concentrations of S1P was added into three replicate wells every 48 h. After incubation at 37℃ in an atmosphere containing 5% CO_2 for 72 h, the number of living cells was calculated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, a MTT solution was added into each well and the cells were incubated for an additional 4 h. The number of living cells was then counted by measuring the absorbance at 490 nm.

Migration assay

Migration assay was performed on a 6.5 mm-diameter transwell chamber with an 8 μm pore size (Corning, NY, USA). The underside of transwell membranes was precoated with fibronectin (10 μg/mL) and allowed to dry. Cells at 70%-80% confluence were serum starved overnight, detached from culture plates by 0.02% EDTA, washed and resuspended in RPMI 1640 medium with 0.1% fatty acid-free BSA at 1×10^6 cells/mL. For migration assay, cells (1×10^5 cells/well) were added into the upper chamber of transwell chambers separated by inserts with 8 μm pores in the presence or absence of S1P. Following an 8-h incubation, nonmigrating cells on the upper surface of membranes were removed with cotton swabs, fixed and stained with 0.1% crystal violet in phosphate-buffered saline (PBS) at the ambient temperature, and then destained with PBS. Migrating cells were counted in 5 randomly selected high-power fields per membrane under an Olympus IX71 inverted microscope.

Statistical analysis

Data are expressed as mean ± SD and shown as error bars in the figures. Statistical comparisons were made by Student's *t*-test. One-way ANOVA with a *post hoc* Bonferroni testing was employed in case of multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of S1P receptors in normal human esophageal mucosal epithelium and Eca109 cell line

Semi-quantitative RT-PCR of mRNA was performed to observe which S1P receptors are expressed in Eca109 cell line and normal human esophageal mucosal epithelium. For comparison, data were also normalized to the expression of the reference gene GAPDH, which gave similar results. Four S1P receptors were found in normal human esophageal mucosal epithelium with the following rank order of mRNA abundance: S1P1>S1P5>S1P3>S1P2. In contrast to Eca109 cell line, the mRNA abundance in S1P receptors was S1P3>S1P1>S1P5>S1P2. S1P4 expression was absent or minimal in Eca109 cell line and normal human esophageal mucosal epithelium (Figure 1). Eca109 cell line expressed S1P3 at a higher level than normal esophageal mucosal epithelium. In contrast, normal

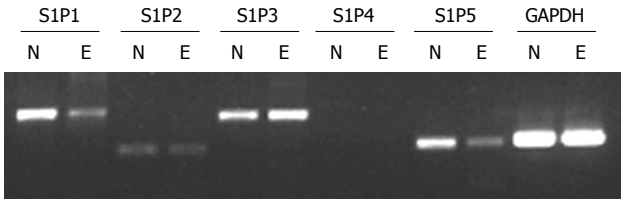

Figure 1 Expression of sphingosine 1-phosphate (S1P) receptors in normal human esophageal mucosal epithelium and Eca109 cell line. N: Normal human esophageal mucosal epithelium; E: Eca109 cell line.

esophageal mucosal epithelium expressed S1P1 and S1P5 at a higher level than Eca109 cell line. On the basis of these results, S1P5 was chosen for further study.

Generation of S1P5 overexpressing Eca109 cell line

Eca109 cells transfected with the S1P5-EGFP or Control-EGFP constructs were cultured in a medium containing 10% FBS. Their localization was visualized by fluorescence microscopy. As shown in Figure 2, control-EGFP was localized at the cytosol of Eca109 cells. In contrast, the S1P5-EGFP was localized at the plasma membrane. Interestingly, control-EGFP-transfected cells displayed the characteristic Eca109 cell morphology. In contrast, S1P5-EGFP-transfected cells displayed spindle cell morphology with elongated and extended filopodia-like projections in a medium containing 10% FBS or 0.1% fatty acid-free BSA.

Effect of S1P on proliferation of control-EGFP or S1P5-EGFP expressing Eca109 cells

Since S1P regulates the proliferation of many cell types, including cancer cells, it is of great interest to investigate whether S1P5 or S1P binding to S1P5 has an effect on the proliferation of control vector and S1P5 transfected Eca109 cells. To assess induction of proliferation, stably transfected cells in 96-well plates containing 10% FBS or 0.1% fatty acid-free BSA were counted after treatment with a vehicle (DMSO) and 10 or 100 nmol/L S1P for 72 h. The number of cells from both control vector and S1P5 transfectants did not increase in response to S1P ($P > 0.05$), suggesting that S1P does not behave as a mitogen at S1P5 (Figure 3).

Interestingly, the proliferation response of S1P5-transfected Eca109 cells was lower than that of control vector-transfected cells in a medium containing 10% FBS (8% lower, $P < 0.05$) or 0.1% fatty acid-free BSA with or without S1P (20%-29% lower, $P < 0.05$ or 0.01), suggesting that S1P5 has an intrinsic activity and inhibits cell proliferation.

Effect of S1P on migration of control-EGFP or S1P5-EGFP expressing Eca109 cells

Migration of Eca109 cells was quantified by transwell-based assay. Transwell membranes were precoated with fibronectin. At a concentration of 10 or 100 nmol/L S1P, the migration of S1P5-EGFP-transfected Eca109 cells was significantly lower than that of control-EGFP-transfected Eca109 cells (48% *vs* 84% at 10 nmol/L S1P, 39% *vs* 94% at 100 nmol/L S1P, $P < 0.001$) (Figure 4A and B). Interestingly, without S1P in the lower chamber, the migrated number of S1P5-EGFP transfected Eca109 cells (145 ± 10) was more than that of control-EGFP-transfected Eca109 cells (51 ± 4, $P < 0.001$) (Figure 4A and C). Both cells failed to migrate on gelatin-coated or non-coated transwell membranes (data not shown). Control-EGFP and S1P5-EGFP transfected Eca109 cells exhibited spontaneous migration without S1P, and the addition of S1P to the lower chamber potently inhibited cell migration.

DISCUSSION

In the present study, normal esophageal mucosal epithelium expressed S1P5 at a high level, and Eca109 cells expressed S1P5 at a low level. S1P5 was predominantly expressed in the white matter tracts and oligodendrocytes, and was particularly abundant in the anterior commissure, corpus callosum, and optic tract. S1P5 mRNA was detected in spleen, peripheral blood leukocytes, placenta, lung, aorta, and fetal spleen. A low level of signals has been detected in many tissues[19,20]. In this study, normal human esophageal mucosal epithelium expressed S1P5 at a high level, suggesting that S1P5 is also a ubiquitous S1P receptor and plays an important role in different tissues and cells.

S1P is believed to be generated intracellularly by all cell types during sphingolipid degradation. Plasma S1P is mainly hematopoietic in origin with erythrocytes as a major contributor to the sources of extracellular S1P, whereas lymph S1P is from a distinct radiation-resistant source[21,22]. The concentration of S1P in plasma and serum is 0.1-0.6 μmol/L and 0.4-1.1 μmol/L, respectively, while the S1P level in tissues is 0.5-75 pmol/mg[23]. Although S1P was originally thought to act as an intracellular second messenger, it has been widely accepted as an intercellular agonist. S1P binding to its receptors activates different intracellular signaling pathways depending on which intracellular Gα protein they couple to. The S1P1 receptor primarily couples through $G\alpha_{i/o}$, whereas S1P2 and S1P3 can couple through $G\alpha_{i/o}$, $G\alpha_{q}$ and $G\alpha_{12/13}$, S1P4 and S1P5 can couple through $G\alpha_{i/o}$ and $G\alpha_{12/13}$[6,7,24-26]. Signaling through $G\alpha_{i/o}$ has been associated with the activation of small guanosine triphosphatase (GTPase) Ras and extracellular signal-regulated kinase (ERK) to promote proliferation, and induction of Pi3K and small GTPase Rac to promote migration. Signaling through $G\alpha_{12/13}$ can promote the activation of small GTPase Rho and Rho-associated kinase (ROCK) to inhibit migration[6]. Furthermore, S1P5 is also a constitutively active receptor with the ability to intrinsically stimulate various effector pathways *albeit* at a different extent in the absence of stimulating ligand[27].

In the present study, normal esophageal mucosal epithelium expressed S1P5 at a higher level than Eca109 cell line. Therefore, Eca109 cells were transfected with the expression constructs of S1P5 or control vector in order to delineate the effects of S1P5 on the growth, migration

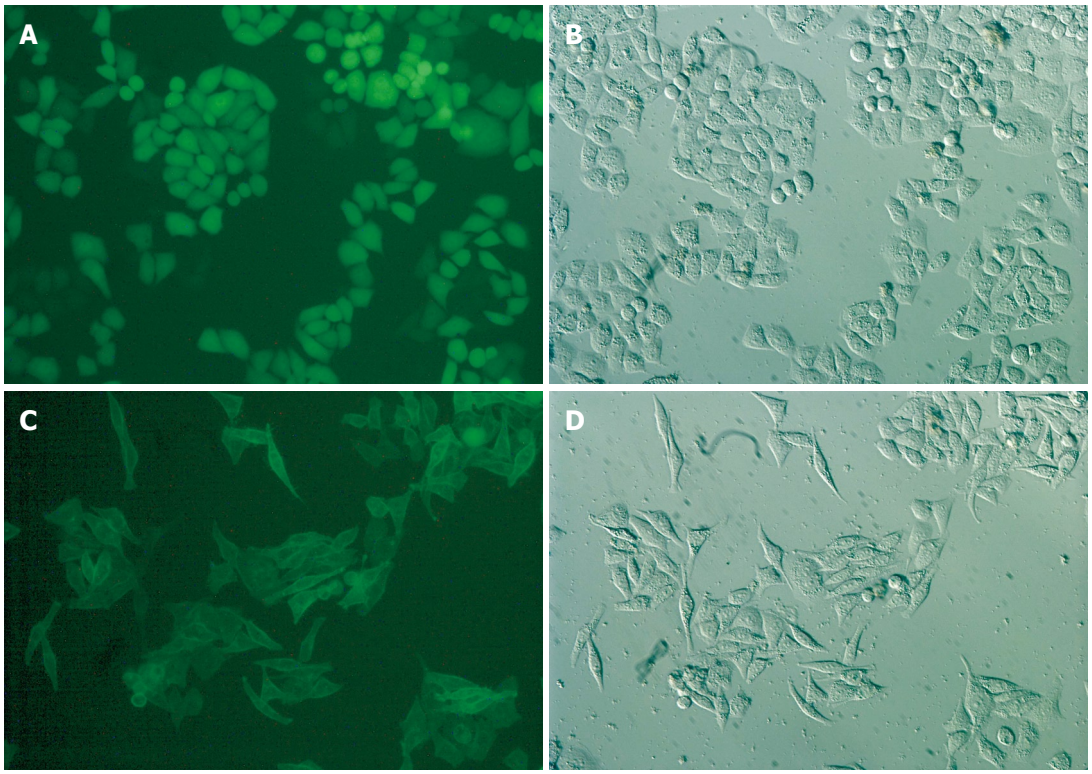

Figure 2 S1P5 receptor overexpression in Eca109 cells causes cell spindle change with elongated and extended filopodia-like projections in a medium containing 10% FBS or 0.1% fatty acid-free BSA (× 200). A, B: Eca109/control-EGFP; C, D: Eca109/S1P5-EGFP.

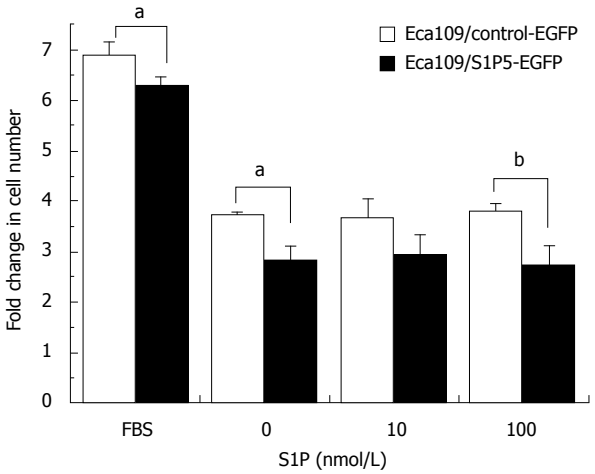

Figure 3 MTT assay showing proliferation of control-EGFP or S1P5-EGFP-transfected Eca109 cells. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

and morphogenesis of esophageal cancer cells. Eca109 cells that overexpress S1P5 were analyzed by phase contrast microscopy. Increased expression of S1P5 induced cell spindle change with elongated and extended filopodia-like projections in a medium containing 10% FBS or 0.1% fatty acid-free BSA. This morphological change is characterized by motile cells. However, transfection of several mammalian cell lines (CHO-K1, HEK293, NIH3T3, RH7777) with the S1P5 receptor induced cell rounding change, which is more pronounced in the presence of S1P-containing serum[27]. Differences in morphological alteration may correlate well with cell type or malignant degree, because CHO-K1, HEK293, NIH3T3 and RH7777 are the normally-derived mammalian cell lines.

In this study, the expression of S1P5 receptor in Eca109 cells reduced the proliferation of Eca109 cells whether or not the medium contained S1P, indicating that the S1P5 receptor is constitutively active in the absence of stimulating ligand. Antiproliferative effect is an intrinsic ability of the S1P5 receptor that is insensitive to ligand stimulation. Analogously, S1P5-transfected CHO cells exhibit an intrinsic activity and inhibit serum-stimulated cell proliferation[28]. S1P5 can negatively and constitutively regulate cellular proliferation by reducing ERK1/2 activity *via* protein-tyrosine phosphatase. $G\alpha_{12/13}$ is a likely candidate G protein for the inhibitory effects of S1P5 on ERK activity. It has been shown that mutation- activated $G\alpha_{12/13}$, but not $G\alpha_q$, $G\alpha_s$, or $G\alpha_{i2}$, can inhibit EGF stimulation of ERK activity in COS-7 cells in a Ras- and Raf-independent manner[29]. Down-regulation of S1P5 in esophageal cancer cells may weaken its antiproliferative effect.

In this study, overexpression of S1P5 receptor constitutively increased the migration of esophageal cancer cells to fibronectin in the absence of S1P, which was consistent with morphological alteration. However, addition of 10 and 100 nmol/L S1P significantly inhibited the constitutive migration of S1P5-transfected Eca109 cells. It has been shown that small GTPases of the Rho family, including Rac, Cdc42, and RhoA, are well-known regulators of cell migration[5,30]. Cell migration induced by S1P receptors (S1P1 and S1P3) involves $G\alpha_{i/o}$-mediated activation of phosphatidylinositol 3-kinase (Pi3K) and

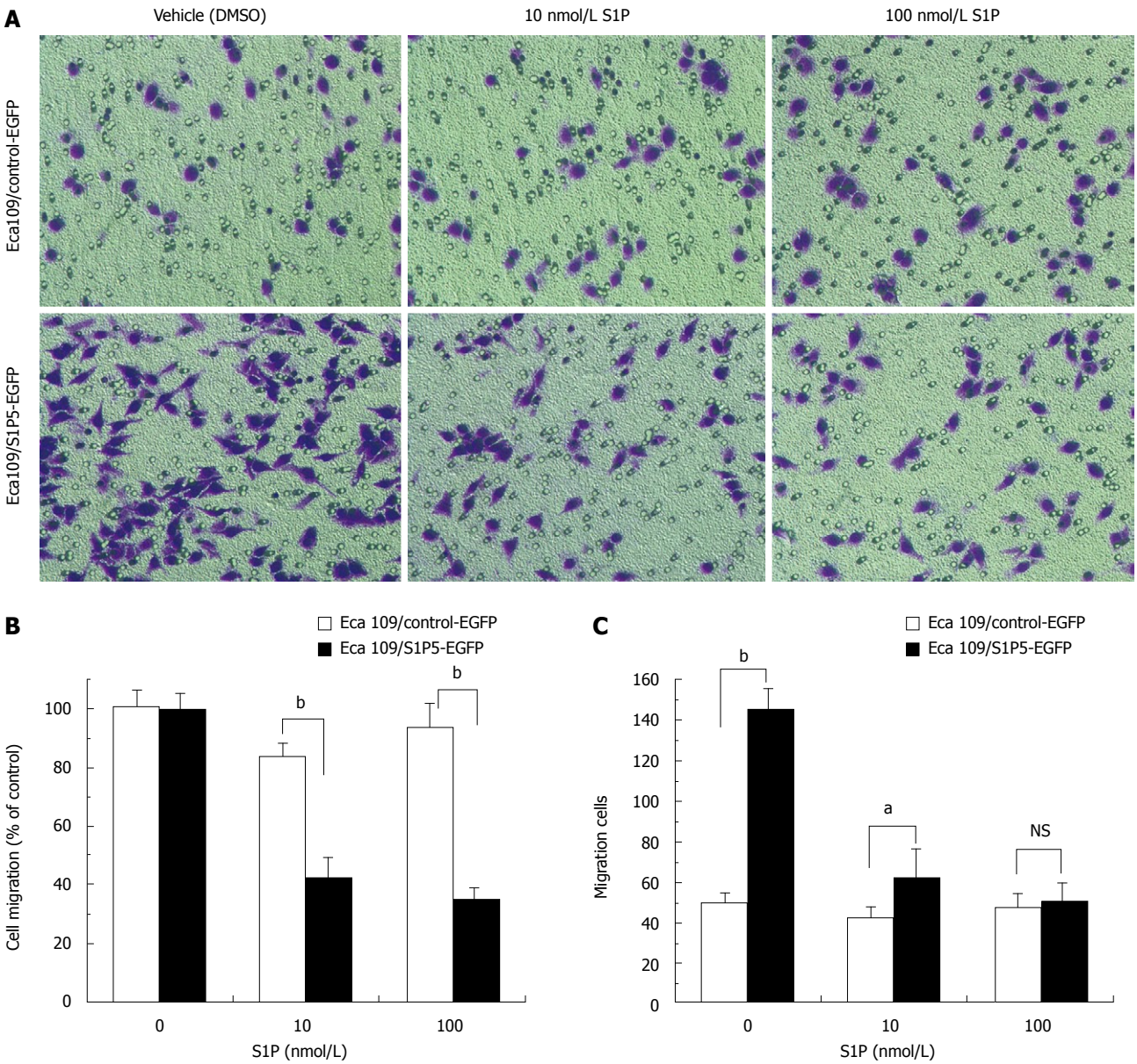

Figure 4 Effect of S1P on migration of S1P5-EGFP-transfected Eca109 cells and control-EGFP Eca109 cells. A: Eca109 cells are allowed to migrate for 8 h, and stained with crystal violet (× 200); B: Results are expressed as percentage in cell migration of S1P-treated cells compared to vehicle (DMSO)-treated cells; C: Results are expressed as migration cell number. $^aP < 0.05$, $^bP < 0.001$ indicate a statistically significant effect between S1P5-EGFP and control-EGFP transfected Eca109 cells treated by indicated concentrations of S1P or vehicle. NS: Not significant.

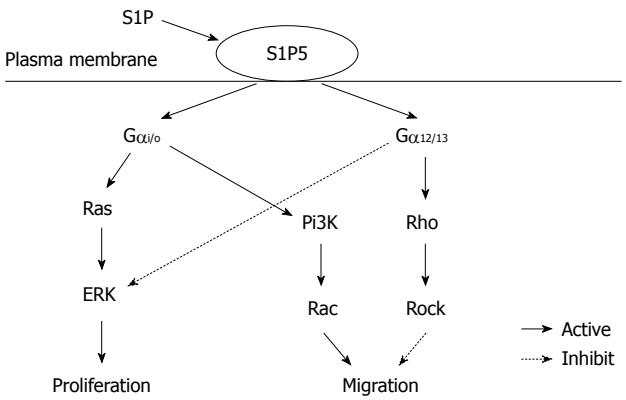

Figure 5 Putative signaling pathway of S1P5.

Rac. S1P receptors (S1P2 and S1P5) mediate inhibition of cell migration by $G\alpha_{12/13}$-evoked stimulation of Rho[12]. It was reported that fibronectin binding to $\alpha_v\beta_3$ integrin can decrease Rho activity and stimulate cell motility and spreading, suggesting that enhanced Rho activity may reduce integrin-driven cell migration[7,31]. Our data support that S1P5 may constitutively induce cell migration due to $G\alpha_{i/o}$-activated Rac and inhibit cell migration due to $G\alpha_{12/13}$-stimulated Rho. In a microenvironment containing S1P interstitial fluid, esophageal cancer cells may escape the inhibitory effect of S1P-S1P5 on their migration by down-regulating the expression of S1P5. The putative signaling pathway of S1P5 is shown in Figure 5.

In summary, S1P5 is a constitutively active receptor

with the ability to intrinsically inhibit cell proliferation and induce cell migration. Cell migration can be inhibited by stimulating extracellular S1P. In normal esophageal mucosa cells, a high expression level of S1P5 may inhibit cell proliferation and migration while esophageal cancer cells may down-regulate the expression of S1P5 to promote cell proliferation and escape the inhibitory effect of S1P-S1P5 on cell migration. Our study is the first to show that the deficiency in inhibitory effect of S1P-S1P5 is of importance in the growth and metastasis of esophageal cancer cells. Further study is needed on the role of S1P and S1P5 receptor in esophageal cancer.

COMMENTS

Background

Esophageal cancer is one of the most common malignancies worldwide. Its mortality is very high due to relatively late diagnosis and inefficient treatment. The ability to reverse the outcome of esophageal cancer is limited due to a poor understanding of its biology. Progression of esophageal cancer may be associated with sphingosine 1-phosphate (S1P) and its receptors S1P1-5, which play an important role in other cancers.

Research frontiers

S1P is a pleiotropic phospholipid mediator. S1P binding to its 5 receptors S1P1-5 mediates diverse cellular responses such as cell proliferation, differentiation, migration, adhesion, and morphogenesis. Recent studies have shown that S1P and its receptors are related with cancer growth and metastasis. In this study, esophageal squamous cell carcinoma (ESCC), S1P receptor expression pattern, and the role of S1P receptors in proliferation and migration of ESCC cells were studied.

Innovations and breakthroughs

Recent reports have highlighted the importance of S1P and its receptors for the growth, metastasis and infiltration of tumors, such as gastric cancer, thyroid cancer, ovarian cancer, and glioma. This study showed that deficiency in inhibitory effect of S1P-S1P5 may be of importance in the growth and metastasis of esophageal cancer.

Applications

The results of this study indicate that deficiency in inhibitory effect of S1P-S1P5 may be of importance in the growth and metastasis of esophageal cancer. S1P5 or its associated signaling molecules may serve as a future strategy in biotherapy for esophageal cancer.

Terminology

S1P is a bioactive lipid that regulates central cellular processes such as cell proliferation, differentiation, migration, adhesion, and morphogenesis. S1P is present in plasma and serum at high nanomolar concentrations. S1P binding to related G-protein coupled receptors is designated as S1P1-5. S1P1, S1P2, and S1P3 receptors which are ubiquitously expressed, whereas S1P4 is restricted to lymphoid tissues and platelets and S1P5 is predominantly expressed in the central nervous system. Binding of S1P to its receptors activates different signaling pathways *via* the heterotrimeric G proteins.

Peer review

The paper is well written and presents an interesting aspect of the pathophysiology of esophageal mucosa which is related to the evolution of esophageal cancer.

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BRIEF ARTICLE

Incidence and risk factors for the development of anemia following gastric bypass surgery

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Abstract

AIM: To evaluate the incidence and risk factors for the development of anemia after Roux-en-Y gastric bypass (RYGB).

METHODS: A retrospective analysis of patients undergoing RYGB from January 2003 to November 2007 was performed. All patients had a preoperative body mass index > 40 kg/m^2. A total of 206 patients were evaluated. All patients were given daily supplements of ferrous sulfate tablets for 2 wk following their operation. Hematological and metabolic indices were routinely evaluated following surgery. Patients were followed for a minimum of 86 wk.

RESULTS: There were 41 males and 165 females with an average age of 40.8 years. 21 patients (10.2%) developed post-operative anemia and 185 patients (89.8%) did not. Anemia was due to iron deficiency in all cases. The groups had similar demographics, surgical procedure and co-morbidities. Menstruation (P = 0.02) and peptic ulcer disease (P = 0.01) were risk factors for the development of post-operative anemia.

CONCLUSION: Iron deficiency anemia is frequent. RYGB surgery compounds occult blood loss. Increased ferrous sulfate supplementation may prevent iron depletion in populations at increased risk.

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Key words: Anemia; Complication; Gastric bypass surgery; Obesity

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INTRODUCTION

Morbid obesity is one of the most common causes of morbidity and mortality in Western countries, and its incidence is rising with unpredictable consequences. It has been estimated that in the next 20 years, 40% of the population in the United States will be morbidly obese with a body mass index (BMI) greater than 30 kg/m^2[1].

Roux-en-Y gastric bypass (RYGB) has become a common procedure for achieving short- and long-term weight loss. It has gained great popularity among surgeons and patients in recent years. Long-term complications are still being discovered, and a variety of nutritional problems have been reported[2]. Because of the altered anatomy, absorption of iron from the proximal

gastrointestinal tract is impaired. Anemia develops in some patients with inadequate oral supplementation or chronic occult blood loss. The purpose of this study is to identify the incidence and risk factors of anemia in morbidly obese patients who underwent RYGB.

MATERIALS AND METHODS

Patients

After approval by the Institutional Review Board of Beth Israel Medical Center, a retrospective review of 206 medical records of patients who had undergone RYGB for the treatment of morbid obesity from January 2003 to November 2007 was conducted. Information gathered included demographic characteristics (age, gender, and ethnicity); BMI; past medical and surgical history; relevant laboratory values (pre- and post-operative levels of serum hemoglobin (Hg), hematocrit, mean corpuscular volume, iron, ferritin, and total iron binding capacity); length of bypass; and degree of weight loss after surgery.

One surgeon (IML) performed all gastric bypass operations using the same operative technique. All patients included in the study underwent standard RYGB surgery with the biliopancreatic limb measured to be 70 + BMI (in cm). The alimentary limb was 75 cm in length. Post-operatively, all patients were given a standard chewable multivitamin (Centrum Chewable, Wyeth Consumer Health Care) and ferrous sulfate tablets, 320 mg daily, starting on the 12th d after the operation. All patients were followed closely by a team of dietitians, bariatric surgeons, and psychologists, both before and after the operation. Laboratory data for hematologic and metabolic indices were obtained at frequent intervals post-operatively. Anemia was defined as serum Hg < 11 g/dL in men, and < 10 g/dL in women. Patients with epigastric pain and/or anemia underwent diagnostic upper gastrointestinal endoscopy. Marginal ulcer was treated with sucralfate slurry and oral proton pump inhibitors for a minimum of 2 wk and patients were reexamined endoscopically 6 wk later.

Statistical analysis

Statistical analysis using the Student *t*-test was used to compare pre- and post-operative serum levels of Hg, iron, and total iron binding capacity, and the χ^2 test was utilized to compare categorical data. The level of statistical significance was set at $P \leqslant 0.05$.

RESULTS

Of 206 patients analyzed, 41 (19.9%) were men and 165 (80.1%) women, with a mean age of 40.8 years (range: 18-60 years). A total of twenty-one (10.2%) patients developed anemia at some point during the post-operative period (Figure 1). Following statistical analysis, patients with the greatest risk for anemia were menstruating females ($P = 0.02$) and patients found to have marginal ulcer on endoscopy ($P = 0.01$). In all cases the anemia was due to iron deficiency (low serum ferritin, elevated total iron binding capacity, and low mean corpuscular volume). Table 1 shows the associated co-morbidities in the patients. The mean values of serum Hg and iron pre- and post-operatively (at 18, 44, 51, and 86 wk after the operation) for all patients are shown in Figure 2A and B. In the immediate post-operative period, serum Hg increased compared to the pre-operative state, and then decreased slowly over time. In contrast, serum iron dropped significantly in the immediate post-operative period, and then rose gradually with oral supplementation.

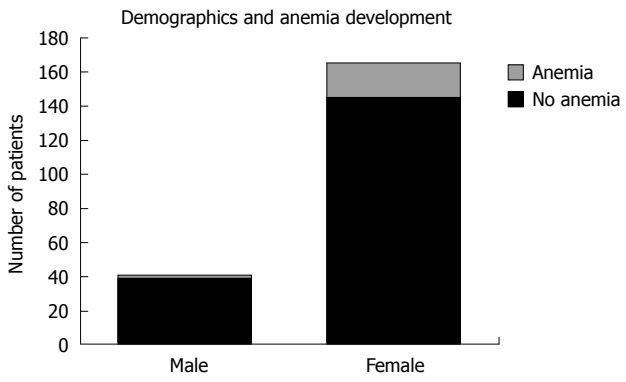

Figure 1 Number of patients who developed post-operative anemia.

Table 1 Associated co-morbidities in the patients who did not develop anemia compared with those who did develop anemia in the post-operative period

Co-morbidity	% of patients without anemia	% of patients with anemia	*P*
Hypertension	81.1	79.5	0.72
Diabetes mellitus	24.2	21.0	0.71
Asthma	29.5	19.8	0.89
Smoking	49.0	44.1	0.86
Menstruation	67.7	84.2	0.02
Peptic ulcer disease	6.0	22.0	0.01
Coronary artery disease	7.9	14.2	0.75
Thyroid disease	13.2	5.6	0.19

DISCUSSION

Nutritional deficiencies following RYGB have been previously reported. Vitamin deficiencies[3], disorders of calcium[4] and copper homeostasis[5] have been reported elsewhere. Iron deficiency anemia presents a special clinical challenge. Disturbing behaviors such as pica (eating of non-food substances) and pagophagia (excessive ice-eating) have been observed in patients with anemia following gastric bypass surgery[6,7]. Severe iron deficiency anemia may require parenteral injection of iron or even packed red blood cell transfusion[8].

Anemia after RYGB may be multi-factorial; resulting from impaired absorption due to the surgically altered gastrointestinal tract, inadequate oral intake, or due to occult blood loss. Anemia may result from bleeding due to the operation itself, such as oozing from the staple or suture lines, marginal ulcers, gastritis, and anastomotic bleeding or due to malabsorption of compounds im-

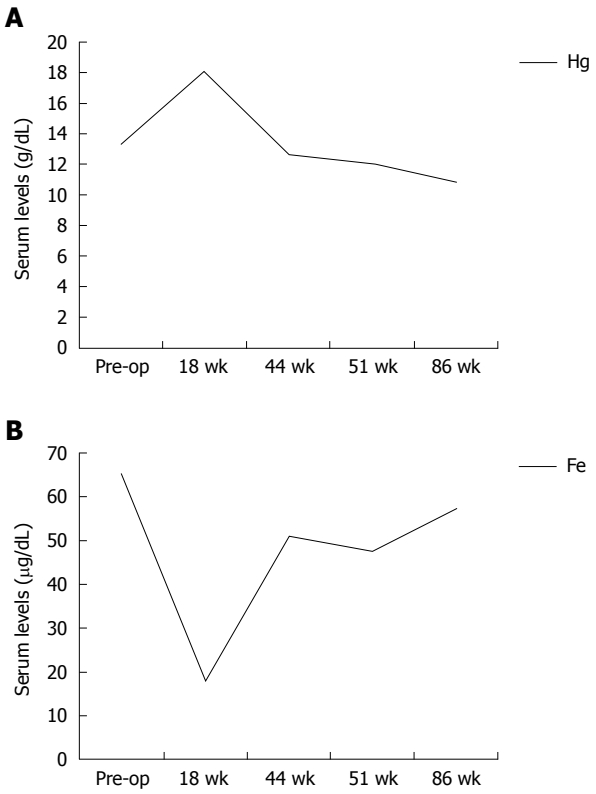

Figure 2 Serum hemoglobin (Hg) (A) levels (g/dL) and iron (Fe) (B) levels (μg/dL) pre- and post-operatively.

portant for the metabolism of Hg, such as iron, folate, thiamine, vitamin B_{12}, niacin, riboflavin, vitamin C, zinc, and copper[1,9]. Diminished intake of red meats (a major natural source of iron) after gastric bypass surgery may further contribute to iron deficiency in these patients[10]. The most prevalent type of anemia is iron deficiency, since iron is absorbed by the duodenum, and this type of anemia results from direct malabsorption due to exclusion of the duodenum from orally ingested nutrients. In addition, the food bolus does not encounter normal amounts of gastric acid secreted by the distal stomach which results in impaired conversion of ingested ferric iron to absorbable ferrous iron[9]. Although prophylactic multivitamin supplements are routinely prescribed for RYGB patients, there are limited data in the medical and surgical literature demonstrating the efficacy of these supplements in the prevention of anemia after gastric bypass surgery[11].

Symptoms of iron deficiency anemia may be nonspecific but include fatigue and muscle weakness, dyspnea, and chest discomfort[12]. Typical laboratory findings include low serum ferritin, elevated total iron binding capacity, low mean corpuscular volume, and decreased intracellular Hg concentration. As such, iron deficiency anemia is typically hypochromic and microcytic[13].

The results of the present study show that 10.2% of our patients developed iron deficiency anemia after RYGB, which is lower than the published data[14]. It has been reported that anemia correlated best with serum transferrin saturation[15]. Coupaye *et al*[8] also reported an incidence of anemia in 10% of patients. The present study demonstrates that risk factors for the development of iron deficiency anemia following RYGB are menstruation ($P = 0.02$) and peptic ulcer disease ($P = 0.01$). It is important to note that these patients developed anemia at some point during the post-operative period, despite being on standard iron supplementation. This may indicate that the current iron supplementation regimens used may not be adequate to prevent the development of iron deficiency anemia in certain patients after RYGB surgery. Love and Billett[16] suggested that the risk of anemia following bariatric surgical procedures continues for life. The incidence of anemia does not appear to be greater following duodenal switch operations[17].

Brolin *et al*[11] found that multivitamin supplements lowered the incidence of folate deficiency, but did not prevent iron or vitamin B_{12} deficiency. However, the authors did not suggest the prophylactic supplementation of vitamin B_{12}, simply because the patients did not develop clinically important deficiency of this vitamin post-operatively. On the other hand, they found that iron deficiency is very resistant to oral iron supplementation, especially in menstruating women, leading them to prescribe prophylactic high doses of oral iron (50 mg or more)[11]. Varma *et al*[18] found that menstruating females were more likely to require parenteral iron therapy after bariatric surgery. Other authors have suggested the addition of vitamin C to oral iron supplementation, but without great success[19]. Previous reports suggest early parenteral iron replacement therapy in patients who do not respond to oral iron therapy in order to prevent anemia-related complications and to maintain patients' quality of life[2,17]. In a prospective, double-blind, randomized study, Brolin *et al*[20] found that prophylactic oral supplementation of 320 mg iron twice daily consistently prevented iron deficiency in menstruating women after RYGB.

In conclusion, it is characteristic that iron deficiency anemia secondary to malabsorption may not start until 2-3 mo after RYGB and can be present up to 2 years later[1] as well as refractory to oral supplementation[20]. As a result, it is of utmost importance that a protocol of long-term follow-up and close biologic and metabolic monitoring of patients after RYGB and the need for a more aggressive therapeutic approach when anemia has been diagnosed is established. Increased ferrous supplementation may be necessary to prevent iron depletion in populations at increased risk of developing iron deficiency anemia after RYGB surgery, such as menstruating women and patients with peptic ulcer disease.

COMMENTS

Background

With the wide application of bariatric surgery in young patients, long-term follow-up is necessary. Iron deficiency and other causes of anemia are prevalent in a large percentage of patients.

Research frontiers

Females of childbearing age and patients with peptic ulcer disease are at the greatest risk for anemia following gastric bypass surgery.

Innovations and breakthroughs

Routine blood work with regular complete blood counts and serum iron levels are required to prevent severe anemia, which may become refractory to oral supplementation.

Applications

Patients with anemia should have endoscopic evaluation to determine possible peptic ulcer disease or other etiologies. Women with heavy menstrual flow should be counseled about the need for routine supplementation.

Terminology

Bariatric surgery is performed for patients with morbid obesity (body mass index greater than 40 kg/m^2 or greater than 35 kg/m^2 for patients with weight-related morbidities) hypertension, non-insulin diabetes or obstructive sleep apnea.

Peer review

This paper looked for the anemia commonly encountered problem after bariatric surgery.

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BRIEF ARTICLE

Ischemic preconditioning-induced hyperperfusion correlates with hepatoprotection after liver resection

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Abstract

AIM: To characterize the impact of the Pringle maneuver (PM) and ischemic preconditioning (IP) on total blood supply to the liver following hepatectomies.

METHODS: Sixty one consecutive patients who underwent hepatic resection under inflow occlusion were randomized either to receive PM alone (n = 31) or IP (10 min of ischemia followed by 10 min of reperfusion) prior to PM (n = 30). Quantification of liver perfusion was measured by Doppler probes at the hepatic artery and portal vein at various time points after reperfusion of remnant livers.

RESULTS: Occlusion times of 33 ± 12 min (mean ± SD) and 34 ± 14 min and the extent of resected liver tissue (2.7 segments) were similar in both groups. In controls (PM), on reperfusion of liver remnants for 15 min, portal perfusion markedly decreased by 29% while there was a slight increase of 8% in the arterial blood flow. In contrast, following IP + PM the portal vein flow remained unchanged during reperfusion and a significantly increased arterial blood flow (+56% *vs* baseline) was observed. In accordance with a better postischemic blood supply of the liver, hepatocellular injury, as measured by alanine aminotransferase (ALT) levels on day 1 was considerably lower in group B compared to group A (247 ± 210 U/I *vs* 550 ± 650 U/I, $P < 0.05$). Additionally, ALT levels were significantly correlated to the hepatic artery inflow.

CONCLUSION: IP prevents postischemic flow reduction of the portal vein and simultaneously increases arterial perfusion, suggesting that improved hepatic macrocirculation is a protective mechanism following hepatectomy.

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Key words: Ischemic preconditioning; Reperfusion injury; Liver; Surgery; Liver blood flow

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INTRODUCTION

The common strategy to reduce intraoperative blood loss in human liver resection consists of temporary clamping

of the portal triad (Pringle maneuver, PM)[1]. The extent of bleeding during surgery is associated with higher postoperative complication rates[2], and the need for autologous blood transfusion may correlate with earlier recurrence of malignancies[3]. The length of the ischemic time strongly determines the release of liver enzymes after hepatectomy[4] indicating considerable hepatocellular injury caused by PM[5]. After declamping of the portal triad, reperfusion of the remnant liver causes additional damage to its parenchymal and non-parenchymal cells[6] which may cause the loss of functional integrity and consecutive hepatic failure[7,8].

Several strategies against the deleterious ischemia-reperfusion (I/R)-induced complications have been suggested[9-11], but these were not routinely introduced in the field of hepatic surgery in humans. This is mainly because of the complex mechanisms involved in I/R, including metabolic, immunological, and microvascular changes which exhibit numerous interactions, rendering the liver a difficult target for preventive strategies.

A new experimental approach to reduce I/R-related injury in the myocardium was firstly presented by Murry[12] who referred to "ischemic preconditioning" (IP) as an adaptation of the myocardium to ischemic stress induced by repetitive short periods of ischemia and reperfusion. Meanwhile, it could also be demonstrated that IP prior to sustained warm ischemia can protect parenchymal and non-parenchymal liver cells by increasing the tolerance against I/R-related organ hypoxia under experimental as well as clinical conditions[13-16]. An important observation was also that IP in human liver surgery was associated with better intraoperative hemodynamic stability, particularly on reperfusion of warm ischemic livers[17].

Although the underlying protective mechanisms of IP are still not fully understood[18], some studies have shown that the activation of Kupffer cells, leucocytes and the release of cytotoxic mediators on reperfusion may lead to a substantial breakdown of the hepatic microcirculation, an event which seems to play a key role following warm and cold ischemia[19-21]. For example, Klar *et al*[22] observed an inverse correlation between the hepatic microvascular blood flow rate and the maximum postoperative enzyme release from the liver. On the other hand, in healthy livers, a balanced portal vein (PV) and hepatic artery (HA) inflow are significantly dependent on the arterial buffer response, an autoregulation system which influences the whole blood supply to the liver at the level of the hepatic arterioles and portal venules, and which is assumed to be predominantly the result of adenosine action[23,24].

At this time, there is evidence that IP improves the hepatic microcirculation after warm as well as cold ischemia, but the influence of PM and IP on macrocirculatory parameters have not been elucidated as yet. In this study, we therefore investigated the hepatic inflow in patients undergoing liver resections with special regard to postischemic liver injury and patient outcome.

MATERIALS AND METHODS

Patients and randomization

The study was approved by the local Ethics committee and written informed consent was obtained from each patient before randomization. We investigated 116 consecutive patients at our institution who were subjected to liver resection (time period 12 mo). Of these, only 68 patients could randomly be assigned according to the inclusion criteria. These were defined as "significant" hepatectomies, i.e. removal of at least one segment. A total of 48 patients were excluded from randomization for the following reasons: (1) extent of liver resection less than one segment according to Couinaud (n = 16); (2) anticipated necessity of total vascular exclusion (n = 8); (3) necessity of additional surgical procedures such as bilioenteric anastomosis or associated gastrointestinal procedures (n = 3); (4) laparoscopic liver resection (n = 10); (5) underlying liver cirrhosis (n = 9); and (6) emergency surgery (n = 2). Of the 68 randomized patients, 7 were withdrawn from the analysis because of intraoperative detection of inoperability. Finally, 61 patients were randomized to a control group (A, n = 31), receiving PM, and to a study group (B, n = 30), who received IP by cross-clamping the portal triad for 10 min followed by 10 min of reperfusion prior to PM. Determination of blood flow of the common HA and PV was carried out simultaneously before starting PM or IP (baseline) as well as 10 min after IP (only group B), and at 15 min of reperfusion as well as before abdominal closure (group A, 32 ± 4 min; group B, 29 ± 6 min after declamping the portal triad) using the transit-time flowmeter (CardioMed CM 2005; MediStern AS, Oslo, Norway). This device measures the difference in travel time between pulses transmitted in the direction of, and against, the flow. The blood flow velocity is directly proportional to the measured difference between upstream and downstream transit times. Because the cross-sectional area of the probe/vessel was known as the probes were individually adapted to the vessel diameter, the product of that area and the flow velocity provided a measure of volumetric flow. The calculations were easily performed by a microprocessor-based converter and displayed online on a computer during surgery.

Study design

The targeted endpoints were the occurrence of IP- and PM-related flow changes of the HA and PV at defined time points. Secondary endpoints were serum levels of alanine aminotransferase (ALT) on postoperative day 1 and complication rates. Operations were performed by 4 experienced abdominal surgeons in a routine clinical setting. Transection was started immediately after inducing PM which was maintained until the transection was finished. Parenchymal transection was performed using a water jet cutter (Saphir Medical, Lyon, France). The volume of the resected liver was determined by the quantity of displaced fluid in a pre-filled trough.

All anesthetic procedures were performed by the same team of experienced anesthesiologists ensuring a standardized protocol. To meet intraoperative fluid demand and to compensate for blood loss, crystalloids and colloidal solutions, respectively, were infused as described elsewhere[17]. Adequate mean arterial pressure (MAP >

Table 1 Baseline data of study patients

Group	Age (yr)	Sex (*n*) M/F	Tumor (*n*) mal/non mal	Fibrosis (*n*) none/mod/sev	Steatosis (*n*) none/min/mod/sev
Control (A) (*n* = 30)	57 ± 14 (26-81)	18/12	26/4	10/15/5	8/16/5/1
IP (B) (*n* = 31)	55 ± 13 (28-77)	19/12	28/3	12/14/5	10/14/5/2
P-value	0.61	0.92	0.65	0.39	0.39

There were no significant differences between the control and ischemic preconditioning (IP) groups. Fibrosis: min (≤ 10%); mod (≤ 40%); liver steatosis: min (≤ 25%); mod (≤ 50%); sev (≥ 50%); mal: malignant. Examination by pathologist of non-cancerous adjacent liver tissue; all patients included.

Table 2 No differences in the intraoperative data of the 2 groups of patients

Group	OP time (min) mean/range	PM time (min) mean/range	LR time (min) mean/range	LV resected (mL) mean/range	Segments resected (*n*) mean/range
Control (A) (*n* = 30)	260 ± 63 (170-420)	34 ± 14 (15-82)	30 ± 10 (10-50)	390 ± 303 (80-1400)	2.7 ± 1.3 (1-5)
IP (B) (*n* = 31)	271 ± 58 (180-420)	33 ± 12 (8-67)	31 ± 11 (15-56)	426 ± 453 (30-2000)	2.7 ± 1.1 (1-5)
P-value	0.36	0.70	0.83	0.77	0.69

OP: Operation; PM: Pringle maneuver; LR: Liver resection; LV: Liver volume.

65 mmHg), central venous pressure (CVP 9-14 mm Hg), and diuresis (> 100 mL/h) were maintained throughout the operation by fluid infusion and, when necessary, by administration of vasopressors (dopamine 2-3 μg/kg per hour and/or norepinephrine) as appropriate.

Laboratory parameters of hepatocellular injury (ALT) and liver function (bilirubin) were obtained before surgery and on postoperative days 1, 2 and 7. Transient liver failure was defined as bilirubin levels > 5 mg/dL and/or prothrombin activity < 40% for at least 3 postoperative days. Fatal liver failure was defined as death from irreversible hepatic dysfunction in the absence of other causes.

Statistical analysis

Numerical values are presented as mean and standard deviation unless otherwise noted. All significance tests were 2-sided and a *P*-value < 0.05 was considered statistically significant. Data analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, USA). Comparison between the 2 groups (with/without IP) was performed using the Mann-Whitney *U* test, the χ^2 test or the Fisher exact test, as appropriate. The association between flow parameters and peak levels of postoperative ALT (day 1) was evaluated by the Pearson Product Moment Correlation. Multivariate analysis of complications was performed by means of logistic regression (backward selection). A multivariate analysis was performed by entering parameters that appeared to be of significance on the univariate analysis into a Cox proportional hazard model to test for significant effects while adjusting for multiple factors.

RESULTS

Baseline data

The analysis of collected data was based on the criteria of the CONSORT group[25]. There were no differences in demographic data and liver histology between the 2 groups (Table 1). Intraoperative parameters were also comparable between the controls and the study population (Table 2).

Flow characteristics

The perfusion data of the HA and PV prior to any intervention (baseline) did not differ between groups (Table 3). Patients who did not receive IP (controls), showed a markedly decreased PV flow by 29% at 15 min reperfusion and by 26% before abdominal closure (32 ± 4 min after declamping). Simultaneously, a slight increase in HA flow of 8% and 3.5% was observed after 15 and 32 min, respectively, of reperfusion of the liver remnants (Figure 1A). In contrast, patients who received IP (group B), maintained stable PV flow during the IP procedure as well as at 15 and 29 min after declamping the portal triad (Figure 1B). In addition, IP induced a more than 200% increase in HA perfusion immediately after IP and the significantly elevated arterial flow was maintained at 15 min (+56%) as well as at 29 min (+38%) after starting reperfusion of the liver remnants, demonstrating a continuing influence of IP on the postischemic blood supply (Table 3, Figure 1B). This results in a total increase in liver perfusion *via* HA and PV of 27% when patients underwent the IP procedure (*P* < 0.01, Table 3).

Laboratory parameters

Postischemic liver damage was measured by ALT levels during the postoperative course. In controls, we observed a significant ALT increase from 28 ± 12 U/L to 550 ± 659 U/L on day 1 when compared to preoperative values, which clearly suggests the PM as the cause of enzyme release. In contrast, IP-treated patients showed markedly reduced ALT levels of about 50% (247 ± 210 U/L, *P* < 0.05), indicating substantial hepatoprotection by this procedure (Table 4). In the further course, postischemic elevated ALT levels in both groups returned to normal within 7 d of hepatectomy. Serum bilirubin levels were determined as a parameter of hepatocellular function, but did not show notably different values at any time during the postoperative course (days 1-7, Table 4).

Intraoperative parameters and postoperative course

Blood loss as well as the need for autologous transfusion

Table 3 Flow characteristics of patients undergoing PM (control, *n* = 30) and IP + PM (study group, *n* = 31)

Group	Vessel	Baseline (mL/min)	After IP (mL/min)	15 min (mL/min)	Before abd. closure (mL/min)
Control	HA	141 ± 24	-	152 ± 29	146 ± 18
IP	HA	126 ± 19	263 ± 51	196 ± 38[a]	174 ± 22[a]
Control	PV	1023 ± 130	-	726 ± 121	757 ± 58
IP	PV	930 ± 94	920 ± 81	919 ± 75[a]	949 ± 62[a]
Total (Co)	HA + PV	1164 ± 72	-	878 ± 77	903 ± 37[c]
Total (IP)	HA + PV	1056 ± 53	1183 ± 64	1115 ± 49[b]	1123 ± 58[b]

There were significantly enhanced PV, HA, and total flows in IP-treated patients. PM: Pringle maneuver; HA: Hepatic artery; PV: Portal vein; abd: Abdomen; Co: Control. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control; [c]$P < 0.05$ *vs* baseline.

Table 4 Outcome data of patients undergoing liver resection with PM (A) or with IP + PM (B)

Group	RPC (units) mean/range	ALT (U/L) mean/range	Bilirubin (mg/dL) mean/range	LF (*n*) transient/fatal[a]	Biliary compl. (*n*) major/minor
Control (A) (*n* = 30)	0.90 ± 1.24 (0-5)	550 ± 650 (54-2888)	1.73 (0.40-9.83)	2 (2)	2[b] (4)
IP (B) (*n* = 31)	0.47 ± 1.31 (0-6)	247 ± 210 (45-852)	1.40 ± 1.26 (0.23-5.59)	0 (1)	1 (2)
P-value	0.014	0.04	0.69	0.04	0.04

[a]Death; [b]Requiring re-operation. Total complication rate was 14/30 (45%) in controls (A) and 6/31 (20%) in the IP group (B). One death in the study group (B) resulted from a perforated duodenal ulcer (postoperative day 21). RPC: Red packed cells; ALT: Alanine aminotransferase; LF: Liver failure; compl.: Complications.

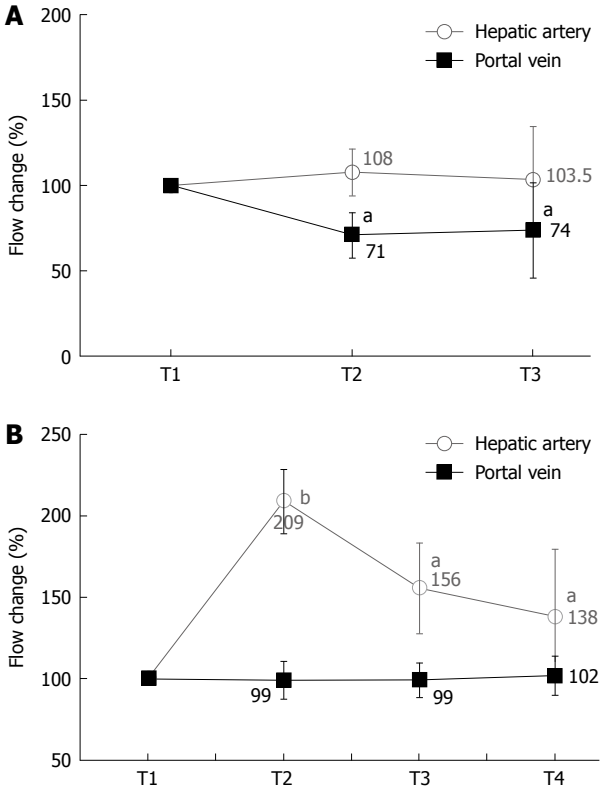

Figure 1 Changes in portal vein (PV) and hepatic artery (HA) inflow (100% = baseline) at operation (mean ± SD) in the control group (A) and study population (B). T1: Before starting the Pringle maneuver; T2: At the end of the IP procedure, i.e. 10 min ischemia and 10 min of reperfusion; T3: One minute of simultaneous reperfusion of PV and HA; T4: Immediately before abdominal closure (29 ± 6 min and 32 ± 4 min after stopping portal triad crossclamping in group A and B, respectively). [a]$P < 0.05$, [b]$P < 0.01$ *vs* baseline.

were significantly lower in the IP-treated group with 17% of patients receiving blood transfusion *vs* 48% in the control group ($P < 0.05$, Table 4). The postoperative course was uneventful in 24/30 (80%) patients in group B but only in 17/31 (53%) patients in group A ($P < 0.05$). Liver dysfunction, as previously defined, occurred in 2 patients of group A, but only in one patient of the IP-treated group (Table 4). Biliary leakage ceased spontaneously in 4 of the 6 patients (67%) of controls, but the other two patients required re-operation and bilioenteric anastomosis. In the study group, 2 patients had transient bile secretion and one patient of this group needed re-operation (bilioenteric anastomosis) (Table 4).

Blood supply to the liver and hepatocellular injury

With regard to earlier work, demonstrating a strong correlation between microcirculatory failure and postischemic enzyme release[22,26], it was of particular interest to determine whether there were changes in macrohemodynamic parameters, i.e. the PM and IP may have an impact on parenchymal cell damage. Firstly, we analyzed the correlation between PV flow and ALT levels on day 1. Interestingly, by applying the Pearson Product Moment Correlation we did not find a significant association between the amount of the hepatocellular injury and quality of PV perfusion, either in controls (r = -0.38, P = 0.3) or in IP-treated patients (r = -0.41, P = 0.2). In contrast, when the HA flow of patients with PM (controls) and the corresponding ALT values on day 1 were analyzed, we found a weak, but significant inverse correlation, indicating a substantial influence of the macrocirculation at reperfusion on postischemic liver injury (r = -0.62, P = 0.042, Figure 2A). This correlation was even more evident, when patients underwent IP prior to PM as shown in Figure 2B (r = -0.73, P = 0.024), suggesting the HA perfusion was more susceptible to the procedure of IP in warm liver I/R.

Predictors of postoperative morbidity

Parameters which were predictive for the development of

Table 5 Factors predicting occurrence of postoperative complications by multivariate logistic regression (backward selection) of factors of importance or significance on univariate analysis

Analysis (*P*-value)	IP treatment	Length of PM	Resected LV	HA flow	PV flow	Liver steatosis	Liver fibrosis
Univariate	0.038	0.043	0.067	0.044	0.72	0.81	0.94
Multivariate	0.047	0.022	NS	NS	NS	NS	NS

Treatment with IP and length of the PM are the only independent predictors of complications. NS: Not significant.

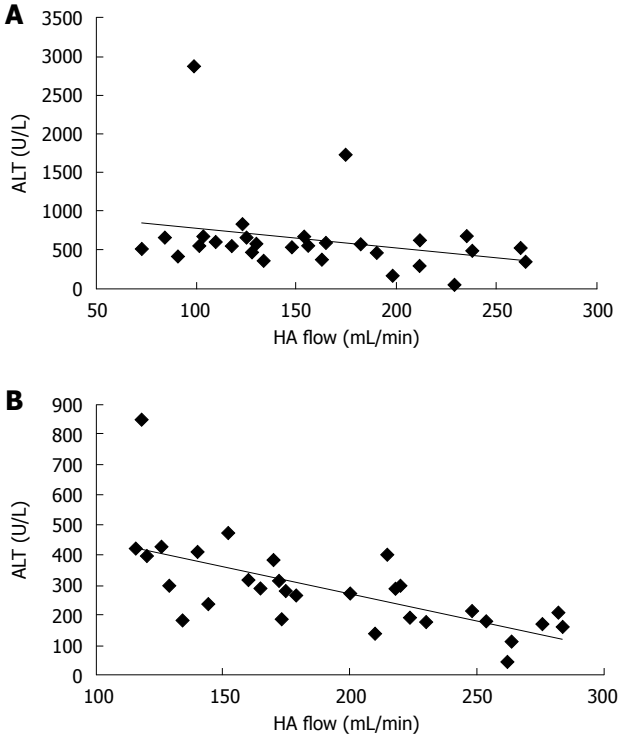

Figure 2 The Pearson product moment correlation between HA flow and alanine aminotransferase (ALT) levels. On day 1, there is an inverse correlation ($P < 0.05$) in the control group (A) undergoing PM ($r = -0.62$). In patients undergoing IP prior to PM (B), an even stronger correlation ($r = -0.73$, $P < 0.01$) was found. Straight lines represent regression analysis; data include values of all patients.

postoperative complications (ALT release, hepatic failure, biliary complications) are given in Table 5. Of 7 investigated parameters only the length of the PM and the procedure of IP were factors independently influencing the outcome of patients after hepatic resection. In particular, it was also found that the quality of HA perfusion was not predictive of complications, presumably reflecting the complex downstream mechanisms of liver I/R which might only in part be related to macrohemodynamic changes of blood flow to the postischemic liver.

DISCUSSION

This study presents the first assessment of human hepatic macrocirculation in response to I/R injury after the PM and IP in human hepatectomy. The main results obtained are: (1) that IP significantly improves hepatic macrocirculation upon reperfusion of liver remnants; (2) that the HA contributes most to the improved blood supply of the liver; and (3) that the quality of HA flow exhibits a strong correlation with postischemic ALT release.

During liver surgery in humans clamping of the portal triad (PM) is practiced to minimize intraoperative blood loss, but can lead to serious liver dysfunction[2,7,27]. Consequently, it has been generally accepted that periods of warm and cold ischemia of the liver should be shortened as much as possible[4,28]. Some experimental and clinical studies suggested that intermittent clamping of the portal triad reduced the negative effects of prolonged continuous warm ischemia on hepatic reperfusion injury[7,29]. However, the most important problems associated with that procedure are an increased blood loss during the episodes of reperfusion[7,30] and a marked prolongation of the operation time[30,31]. Ischemic preconditioning may combine the beneficial effects on reperfusion injury with the avoidance of additional blood loss during surgery[30,32].

In animal experimental studies, hepatic I/R is associated with perfusion failure of sinusoids due to significant hemoconcentration, reduced perfusion pressure, pressure-related sinusoidal leukostasis, as well as sinusoidal narrowing caused by hypoxia-induced endothelial swelling[33]. The impaired restoration of hepatic microvascular flow correlates also with the extent of liver injury after hemorrhagic shock and resuscitation[34]. Prevention of microcirculatory failure may largely protect the liver from parenchymal cell necrosis after I/R, suggesting that the degree of microvascular failure determines the extent of lethal hepatocyte injury[34]. Similar relationships between liver perfusion and injury could be observed in humans after warm and cold ischemia[26,35]. Interestingly, Puhl *et al*[26] could demonstrate a significant inverse correlation of the quality of the microvascular perfusion with postoperative liver enzyme release as well as bilirubin elimination in human liver transplants. This indicates that the observed (relative) hyperemia in the sinusoids might confer protection against postischemic liver injury. Another study by Klar *et al*[22] could also demonstrate in patients an inverse correlation between the intraoperatively measured hepatic microvascular blood flow rate and the maximum postoperative enzyme release from the liver. However, neither study included data on macrohemodynamic parameters, i.e. flows in the HA and PV in these clinical settings. Therefore, it cannot be concluded that the preservation of the sinusoidal blood flow after I/R can be the result of preservation of the HA and PV inflow during the reperfusion period.

While changes in the nutritive sinusoidal blood flow are the result of complex humoral, cellular, and immunologic interactions, the mechanisms leading to microcirculatory shutdown after liver I/R are related, at least in part

also to upstream mechanisms, i.e. regional macrocirculation of the liver[23,36]. Consequently, efforts should also focus on the preservation of an optimal blood supply to the liver under different pathological conditions at the levels of the HA and PV. In line with this, it was demonstrated that hepatosplanchnic blood flow was still reduced in humans at 60 min of reperfusion after severe hypovolemia, even though arterial blood pressure, cardiac output, and blood flow to other organs was already fully restored[37].

In the present study, we observed a severely decreased portal blood flow and a minimal increase in the HA flow on reperfusion of livers undergoing more than 30 min of portal crossclamping, a time period which is known to induce significant hepatocellular injury in humans[16]. We also found that IP could abolish the postischemic PV flow decrease and, *vice versa,* IP induced a significantly better HA flow throughout the reperfusion period, resulting in a markedly improved overall blood supply to the liver. This might be of substantial importance with regard to the nutritive sinusoidal perfusion, given that the autoregulatory capacity of the HA to maintain a constant flow rate in the presence of pathological conditions is limited, known as the hepatic arterial buffer response[23,24]. Therefore, IP effectively restored the total hepatic flow to almost normal values during reperfusion whereas in the control group the minimal increase of the HA flow failed to compensate for the postischemic perfusion deficit at the PV (Table 3). Because hemodynamic parameters, like MAP, CVP, and heart rate were kept stable upon reperfusion, and IP-treated patients did need significantly lower norepinephrine administration to maintain an adequate MAP, this observation can only be explained by an IP- mediated effect[17]. However, the exact mechanisms for that observation cannot be answered in the present study, although it was reported that both the hepatic arterial pressure-flow autoregulation and buffer response are mediated through changes in the washout of locally produced adenosine[38], and that IP may effectively increase the formation of the vasodilator adenosine while the unwanted degradation of adenine nucleotides to purines caused by the PM can be attenuated by IP[39].

Interestingly, we observed also a significant correlation between the postischemic ALT release on day 1 and the HA flow in controls, and this was even more pronounced in IP-treated patients, which clearly demonstrates that local macrohemodynamic changes at the HA may play an important role in the prevention of hepatic I/R injury. Because in our study hepatocellular damage was independent of postischemic PV flow changes, one can only speculate about the relevance of this observation, in particular with regard to the "small for size" problem in living related donor liver transplantation. This phenomenon, referred to as portal venous hyperperfusion of the partial liver allograft and associated with severe transplant dysfunction is thought to be the result of an imbalanced autoregulation of the arterial buffer response, resulting in a low concentration of adenosine-mediated HA branch constriction in the presence of increased portal perfusion[40]. Because hepatectomies with the loss of up to 5 liver segments as in our study may be compared to the above-mentioned situation, it was interesting that we found a substantial decrease of the PV flow upon reperfusion in controls which was not adequately compensated by an increase in the HA flow whereas in IP-treated patients the HA flow was significantly enhanced while the PV flow was kept stable during the reperfusion period, suggesting an impact of IP on the empirically observed reciprocal regulation between PV and HA inflow[37]. However, the previously described substantial alterations of nutritive sinusoidal flow impairments may occur, even in the absence of overt macrohemodynamic changes, as a result of an I/R-induced heterogeneity of hepatic microvascular downstream mechanisms[36,37]. In addition, despite postischemic ALT release being a well- established parameter for the estimation of hepatic injury following I/R, ALT levels alone are not predictive of the occurrence of postoperative liver failure following liver resection or transplantation[14,41].

In summary, this study provides some new insights into macrohemodynamic changes during liver resection under inflow occlusion and on treatment with IP in humans. As we could not simultaneously investigate the hepatic microcirculation in this setting, the impact of HA and PV perfusion alterations on the nutritive blood supply in sinusoids remains speculative. Further studies are necessary to clarify this aspect, and in particular, the impact of macro- and microhemodynamic changes on postischemic liver function in humans.

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COMMENTS

Background

Liver surgery has become a safe procedure in the past years and is mainly done because of malignant tumors. A common strategy to reduce blood loss during surgery is to temporarily shut down the blood supply to the liver [pringle maneuver (PM)], which, however may be associated with severe hepatocellular injury and consequent enhanced morbidity.

Research frontiers

Many efforts were undertaken to overcome the deleterious effects of ischemia-reperfusion injury of the liver caused by the PM. A new method of hepatocellular protection comprises ischemic preconditioning (IP), i.e. an additional short ischemia and reperfusion period prior to sustained ischemia, as set by the PM. However, mechanisms of protection by IP are still largely unknown.

Innovations and breakthroughs

Recent reports have highlighted numerous mechanisms which are involved in the protection of ischemic livers, including humoral, cellular, and immunologic interactions. Furthermore, an improved hepatic microcirculation seems to play a key role in liver protection following IP. However, no data were available which comment on hepatic macroperfusion under different conditions, such as IP.

Applications

By understanding how changes of blood flows of the portal vein and hepatic artery under inflow occlusion or IP may influence hepatocellular damage, this

study may provide some strategies for therapeutic intervention during liver surgery, such as selective portal triad clamping.

Terminology

IP of the liver consists of a short ischemia-reperfusion period (e.g. 10 min/10 min) immediately prior to longer periods of liver ischemia, which are often necessary during extended liver resections. Although it seems paradoxical, this additional short ischemia-reperfusion period confers protection on the liver by different mechanisms.

Peer review

This is a good study from a well-known liver surgery group with excellent methodology and adequate data analysis. The group expanded on their previous investigations on the role of IP in liver protection following resection. In the current study, the authors intuitively studied the effect of IP on hepatic artery and portal vein blood flow and demonstrated that IP prevented postischemic flow reduction of the portal vein and significantly increased the arterial perfusion.

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BRIEF ARTICLE

Comparative analysis of clinicopathological correlations of cyclooxygenase-2 expression in resectable pancreatic cancer

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Abstract

AIM: To perform a comparative analysis of clinicopathological correlations of cyclooxygenase-2 (COX-2) expression in pancreatic cancer, examined by monoclonal and polyclonal antibodies.

METHODS: The COX-2 expression in 85 resection specimens of pancreatic ductal adenocarcinoma was immunohistochemically examined using both monoclonal and polyclonal antibodies. The final immunoscores were obtained by multiplying the percentage of positive cells with the numeric score reflecting the staining intensity. COX-2 expression levels were classified into three categories (0, 1+, and 2+) and the clinicopathological correlations were statistically evaluated and analyzed.

RESULTS: The positive tumor expression rates of COX-2 were 80.5% using monoclonal antibody and 69.4% using polyclonal antibody. In the Kaplan-Meier analysis, no significant correlations were found between levels of COX-2 expression and overall survival (OS), but trends to longer OS were found in COX-2 negative cases using monoclonal antibody. Significantly longer disease free survival was revealed in COX-2 negative cases using monoclonal antibody (P = 0.019). No correlations between COX-2 expression levels and grade (G), tumor (T) status and nodal (N) status were demonstrated. Low histological grade showed a strong association with a longer OS (P < 0.001). Correlation of survival and T status revealed a shorter OS in T3 tumors, but the results reached only marginal statistical significance (P = 0.070). In the multivariate Cox proportional hazards regression model, histological grade, T and N status remained valuable predictors of a worse survival with borderline significance for T [hazards ratio (HR) = 4.18 for G (if G = 3, P < 0.001); HR = 1.64 for T (if T = 3, P = 0.065); HR = 2.53 for N (if N = 1, P = 0.006)]. Higher grade, T or N status was associated with a worse OS.

CONCLUSION: The immunohistochemically assessed level of COX-2 expression does not seem to represent a valuable independent prognostic factor and is not superior to the conventional prognostic factors.

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Key words: Pancreatic cancer; Cyclooxygenase-2; Immunohistochemistry; Monoclonal antibody; Polyclonal antibody

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INTRODUCTION

Invasive ductal adenocarcinoma is the most common neoplasm in the pancreas, constituting about 90% of all pancreatic tumors[1]. Pancreatic cancer represents the fourth leading cause of cancer-related death among men and women in western countries[2,3]. Pancreatic cancer has no early warning signs and symptoms, so most patients present with advanced disease. Despite improved diagnostic and therapeutic modalities, pancreatic cancer still has a very poor prognosis. The incidence of pancreatic cancer almost equals the mortality rate, and it has one of the lowest overall 5-year survival rates (under 5%) among the epithelial cancers[4-6].

The currently accepted model of the development of pancreatic ductal adenocarcinoma (PDAC) understands the oncogenesis of this malignancy as a multistep process, characterized by the progression from the normal ductal epithelium through the spectrum of duct lesions known as pancreatic intraepithelial neoplasia (PanIN) to invasive ductal adenocarcinoma[7,8]. PanINs represent precursor lesions of ductal adenocarcinoma and their classification system distinguishes three grades of PanINs, which harbor a number of well-established molecular events including activation of oncogenes, inactivation of tumor suppressor genes, inactivation of DNA mismatch repair genes, various epigenetic alterations, dysregulation of oncoproteins, and others[9-11]. Mediators of inflammatory pathways [e.g. cyclooxygenase-2 (COX-2), nuclear factor-κB (NF-κB), 5-lipooxygenase (5-LOX), interleukin-8 (IL-8) *etc.*] are also known to play an important role in carcinogenesis of pancreatic cancer and represent a key link between chronic inflammation and cancer[12-14]. Prostaglandin H2 synthase (COX) represents an enzyme which is involved in the conversion of arachidonic acid to prostaglandins. Two COX isoforms, COX-1 and COX-2, have been identified to date[15]. COX-1 is constitutively expressed in many tissues, and is involved in prostaglandin synthesis under physiological conditions. COX-2 is expressed under certain extracellular or intracellular stimuli, including mitogens, growth factors, proinflammatory cytokines, hormones and infectious agents, and is a component of cellular responses to inflammation. COX-2 is overexpressed in many human solid tumors including pancreatic cancer[16,17]. Several studies have suggested a potential involvement of COX-2 pathways in the regulation of tumor-associated angiogenesis and cell growth in pancreatic cancer. COX-2 has been demonstrated to inhibit apoptosis, promote cell proliferation and to induce the expression of vascular endothelial growth factor[18-21]. COX-2 overexpression has been reported in 56% to 90% of ductal adenocarcinomas[22]. A high level of COX-2 overexpression has also been described in PanIN lesions[23-25], and COX-2 was suggested as a potential therapeutic target for chemoprevention and therapy of pancreatic cancer[22,26].

A significant inverse relationship between COX-2 overexpression and survival rates has been reported in retrospective studies of different types of malignancies[27-32]. Conflicting results have been shown in pancreatic cancer[33-39] and the possible role of primary antibody used for the detection of COX-2 expression has been suggested[33,40]. Comparative analysis has not yet been performed. In our study the expression of COX-2 in resectable pancreatic ductal adenocarcinomas was immunohistochemically examined using both monoclonal and polyclonal antibodies, and analyzed and correlated with clinicopathological parameters.

MATERIALS AND METHODS

Study group and tissue specimens

The study group (summarized in Table 1) consisted of 85 patients [41 males (48.2%) and 44 females (51.8%); median age 61 ± 9.5 years (range 39-85 years)] with resectable pancreatic cancer who had undergone pancreatectomy at the Faculty Hospital Brno and the Masaryk Memorial Cancer Institute between 2000 and 2006. Seventy eight patients underwent hemipancreaticoduodenectomy, 5 patients caudal pancreatectomy and 2 patients total pancreatectomy. No distant metastases were found at initial diagnosis (M0). Selected tumors were histologically confirmed to be invasive ductal adenocarcinomas of the pancreas, and in all patients the formalin fixed paraffin embedded tissues were available for immunohistochemistry. Grading of tumor differentiation was done based on WHO criteria combining the judgment of glandular differentiation including mucin production, mitotic count and nuclear features. Tumor staging was performed according to the International Union Against Cancer TNM System (the 6th edition). The follow-up was available for 75 patients, with five patients alive at the end of the study. Survival data for the patients were obtained from the National Oncological Register of Czech Republic. Resection specimens were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin. Hematoxylin-eosin (HE) staining of tissue sections was used to identify representative samples with structures of pancreatic invasive ductal adenocarcinoma (up to three tissue blocks for sectioning were selected for each individual PDAC case). These were selected for immunohistochemical (IH) analysis.

Immunohistochemistry

The results of IH analysis of COX-2 expression using polyclonal anti-COX-2 antibody [rabbit polyclonal antibody against COX-2 (H-62), dilution 1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA] were retrieved from our previous study[38], in which the identical study population was examined.

Table 1 Patient, tumor and treatment characteristics, and levels of COX-2 expression (n = 85)

	n
Gender	
Male	41
Female	44
Mean age (range) (yr)	61 ± 9.5 (39-85)
Location of tumor	
Head, body	80
Tail	5
T category	
T1	6
T2	29
T3	50
N category	
N0	23
N1	62
M category	
M0	85
M1	0
Surgical procedure	
Pancreatoduodenectomy	78
Caudal pancreatectomy	5
Total pancreatectomy	2
Histological differentiation	
Well	8
Moderately	54
Poorly differentiated	23
No adjuvant therapy	30
Adjuvant therapy	33
Chemotherapy	23
Chemoradiotherapy	10
COX-2 expression with monoclonal antibody	
COX-2 negative	16
COX 1+	27
COX-2+	39
COX-2 expression with polyclonal antibody	
COX-2 negative	26
COX 1+	28
COX-2+	31

COX-2: Cyclooxygenase-2.

In the present study, the COX-2 expression was evaluated using mouse monoclonal anti-COX-2 antibody (dilution 1:50, clone CX229; Cayman Chemicals, Ann Arbor, MI, USA). IH detection of COX-2 was performed on 4 μm thick tissue sections applied to positively charged slides. The sections were deparaffinized in xylene and rehydrated through a series of alcohols. Antigen retrieval was performed in the lab microwave (Milestone, Sorisole, Italy) by heating in citrate buffer at pH 6.0 for 20 min at 98℃. The slides were incubated with anti-COX-2 antibodies overnight at 4℃. A streptavidin-biotin peroxidase detection system was used for COX-2 IH using monoclonal antibody (mouse IgG Vectastain Elite Kit, Vector Laboratories, Burlingame, California, USA). The visualization was performed using 3,3'-diaminobenzidine as a substrate (Fluca, Buchs, Swizerland). The slides were counterstained with Gill's hematoxylin.

Sections from COX-2 strongly positive colon carcinoma (immunoscore 2 - see explanation below) were used as positive controls for COX-2 IH in each run. Additionally, COX-2 immunoreactivity in islets of endocrine pancreas served as an efficient internal positive control[23]. Negative controls of COX-2 IH were performed by incubating samples without the primary antibody.

Evaluation of immunostaining

At least three different representative high-power (× 400) fields of tumor infiltration were examined. Cases with no stated minimal amount of the representative tumor tissue available were excluded. For both monoclonal and polyclonal antibody COX-2 IH, the percentage of positive cells was assessed, and the immunostaining intensity was classified into three categories: numeric score 0, no staining; numeric score 1, weak staining; numeric score 2, moderate and strong staining. The final immunoscore was obtained by multiplying the percentages of positive cells with the numeric score reflecting the staining intensity. Immunoscores were categorized into three levels: 0 (immunoscore < 20); 1 (immunoscore 20-49); 2 (immunoscore 50-200). In cases with heterogeneous expression of COX-2, the average score was counted.

Statistical analysis

Association of categorical parameters was analyzed and presented in contingency tables and tested using Pearson Chi-square test. Kaplan-Meier curves were constructed and median survival times were computed for survival data. Log-rank test was used to test differences between groups. Univariate and multivariate analyses of survival data were performed using Cox regression model to evaluate the predictive value of analyzed parameters (COX-2, histological grade, T status, N status). The level of significance was considered $P < 0.05$. All analyses were done using Statistica for Windows 8.0.

RESULTS

Patient, tumor and treatment characteristics, and levels of COX-2 expression are summarized in Table 1. The immunohistochemical analysis of COX-2 expression displayed considerable heterogeneity in staining intensity and percentage of positive cells between and within individual PDAC cases. In cases with heterogeneous COX-2 expression within a lesion, the average immunoscore for such a case was counted. Using both monoclonal and polyclonal antibodies, the PDAC cells showed diffuse cytoplasmic patterns of expression. Expression of COX-2 (Figure 1), examined using monoclonal anti-COX-2 antibody, was revealed in 66 cases (80.5%); 27 tumors (32.9%) expressed COX-2 at 1+ level, 39 at 2+ level (47.6%), and 16 cases (19.5%) were COX-2 negative (in 3 samples, a sufficient amount of neoplastic tissue was not available for IH analysis, and analysis of COX-2 expression using monoclonal antibody was not performed). Results of immunohistochemical analysis of COX-2 expression by polyclonal antibody were retrieved from our previous study[38] and are included in Table 1 [28 tumors (32.9%) expressed COX-2 at 1+ level, 31 at 2+ level (36.5%), and 26 cases (30.6%) were COX-2 negative].

The median overall survival (OS) in the study population was 1.3 years. There was no significant difference between OS in males and females (median OS 1.1 years

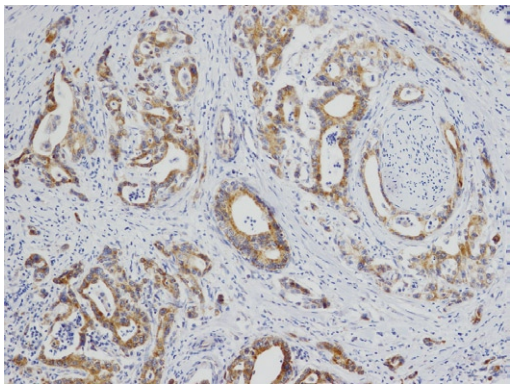

Figure 1 Overexpression of cyclooxygenase-2 (COX-2) in pancreatic invasive ductal adenocarcinoma with displayed perineural spreading of the tumor (COX-2 immunohistochemistry, original magnification, × 100).

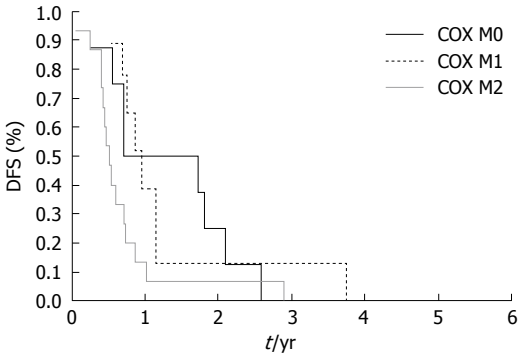

Figure 2 Kaplan-Meier disease free survival (DFS) curves for patients with resectable pancreatic cancer stratified by the level of COX-2 expression examined using monoclonal antibody. High expressors of COX-2 (2+, COX M2) had significantly lower survival curve than low expressors of COX-2 (1+, COX M1) or COX-2 negative cases (0, COX M0), $P = 0.019$.

in males *vs* 1.3 years in females; $P = 0.143$). Regarding nodal (N) status, N1 status was associated with shorter median OS (1.0 year in N1 group *vs* 1.5 years in N0 group; $P = 0.102$). Median OS for patients with adjuvant therapy was 1.4 years, and for patients without adjuvant therapy 1.3 years. The median disease free survival (DFS) for patients with adjuvant therapy was 0.8 years, and for patients without adjuvant therapy 0.7 years. Differences did not reach statistical significance.

No correlations between the levels of COX-2 expression (using both monoclonal and polyclonal antibodies) and the histological grade, T or nodal status were revealed.

In the Kaplan-Meier analysis, no significant correlations were found between the levels of COX-2 expression and OS (again using both polyclonal and monoclonal antibodies). However, trends to longer median OS were found in COX-2 negative cases (1.4 years in COX-2 negative *vs* 1.3 years in low expressors *vs* 1.0 in high expressors) using monoclonal antibody.

Correlating the levels of COX-2 expression and DFS; using polyclonal antibodies, trends to longer median DFS were found in COX-2 negative cases (1 year in COX-2 negative *vs* 0.7 years in low expressors *vs* 0.5 years in high expressors), but these values were not significant ($P = 0.211$); using monoclonal antibody, a statistically significant correlation was found between COX-2 expression levels and DFS. Significantly longer DFS was revealed in COX-2 negative cases (median DFS 1.2 years in COX-2 negative *vs* 0.9 year in low expressors *vs* 0.5 year in high expressors, $P = 0.019$) (Figure 2).

Low histological grade showed a strong association with a longer OS ($P < 0.001$). Median OS for patients with grade 3 tumors was 0.7 years *vs* 1.6 years for grade 2 and 1.8 years for grade 1. Correlation of survival and T status revealed a shorter OS in T3 tumors, but the results reached only marginal statistical significance ($P = 0.070$). In a multivariate Cox proportional hazards regression model, histological grade (G), T, and nodal status remained valuable predictors of a worse survival with borderline significance for T [hazards ratio (HR) = 4.18 for G (if G = 3, $P < 0.001$); HR = 1.64 for T (if T = 3, $P = 0.065$); HR = 2.53 for N (if N = 1, $P = 0.006$)]. Higher grade, T or N status was associated with a worse OS.

DISCUSSION

Immunohistochemistry has become an integral part of histopathological diagnosis and can also provide important data to predict clinical course of the disease and potential therapeutic responsiveness. Thus, a validation of usefulness of different immunoprofiles and methodological approaches in predictive oncopathology is necessary. Based on a PubMed search, there are 7 papers[33-39] reporting the correlations between COX-2 expression and selected clinicopathological parameters, including survival rates, in pancreatic cancer. The possible role of the COX-2 expression profile used as a prognostic factor for pancreatic ductal adenocarcinomas is largely discussed. Five studies did not show any trends or statistically significant correlations between COX-2 expression and survival rates[35-39]. Our previous study[38] also did not show significant correlations between these parameters, although trends to longer DFS in COX-2 negative cases were demonstrated. Recently, Matsubayashi *et al*[34] reported that the COX-2 expression in patients with pancreatic ductal adenocarcinoma undergoing a potentially curative pancreaticoduodenectomy is predictive of survival, independent of other known prognostic markers, particularly in cancers ⩾ 3 cm. These results were supported by Juuti *et al*[33] who reported the expression of COX-2 to be associated with poor outcome for pancreatic ductal adenocarcinoma, which was independent of tumor stage, grade, or age in multivariate analysis. Moreover, these results were obtained, even if not only surgically resectable pancreatic cancers were included for analysis. Both Matsubayashi *et al*[34] and Juuti *et al*[33], who showed the significant correlation between COX-2 expression and clinical outcome, worked with mouse antihuman monoclonal antibodies. A discussion about the possible role of the selected antibody has been initiated. Antigenic blocking experiments showed higher sensitivity but lower specificity of polyclonal antibodies when compared with monoclonal antibodies[40]. The presented comparative analysis only partially supported the possible prognostic role of COX-2 expression. Results retrieved from our previous study[38], in which rabbit polyclonal antibody was

used, were completed with results obtained by examination of COX-2 expression using the same mouse antihuman monoclonal antibody that was used in the studies of Juuti *et al*[33] and Matsubayashi *et al*[34]. Using the monoclonal antibody, a significantly shorter DFS was found in patients with COX-2 positive tumors. Trends to shorter DFS in COX-2 expressors were only reported using the polyclonal antibody[38]. No significant results were obtained regarding OS; the only trends to longer OS were demonstrated in COX-2 negative cases using monoclonal antibody. Our results do not sufficiently support the findings of Juuti and Matsubayashi, even when the standardized protocols for immunohistochemical evaluation of COX-2 expression, which should be preventive of known technical and interpretative pitfalls in immunohistochemistry, were used. Except for the mentioned type of antibody, the main factors potentially affecting immunohistochemical staining are the tissue processing, especially the type and length of fixation, and antigen retrieval. All tissues included in the study were routinely fixed in 10% neutral buffered formalin for 24 h, and antigen retrieval was performed using a standardized protocol. To avoid the misinterpretation of results, especially unspecific immunostaining, systems of positive (both external and internal) and negative controls were used in each run. Based on our results, COX-2 expression does not seem to represent a valuable independent prognostic factor, even if using the monoclonal antibody brought statistically significant results when correlating DFS and COX-2 expression levels and when trends to longer median OS were also demonstrated.

Correlating the COX-2 expression with the level of tumor differentiation, no significant relationship was revealed between the level of COX-2 expression and the histological grade of the pancreatic cancer independently of the antibody used. These findings are in agreement with previous studies[23,35,41]. Merati *et al*[36] described increased expression of COX-2 in well differentiated ductal adenocarcinomas, but this trend was not observed in our study group, and the proposed role of COX-2 in carcinogenesis of pancreatic cancer remains puzzling.

The routinely used grading of the histological differentiation of tumors and evaluation of T and N status were proven to represent efficient prognostic factors. Higher grade of the tumor and N1 status were significantly associated with a shorter survival in patients with resectable pancreatic cancer. T3 status was associated with shorter survival only with marginal significance.

In conclusion, based on these presented results and the previously published data, an immunohistochemical assessment of COX-2 expression is not superior to the conventional prognostic factors such as tumor histological grade, stage, and nodal status. The possible role of COX-2 in potential targeted therapy and chemoprevention of pancreatic cancer using COX-2 inhibitors remain as unanswered questions and need further evaluation.

COMMENTS

Background

Pancreatic cancer is the fourth leading cause of cancer-related deaths. The incidence of pancreatic cancer almost equals the mortality rate and the five-year survival rate does not reach 5%. Mediators of inflammatory pathways are known to play an important role in carcinogenesis and represent a key link between chronic inflammation and cancer.

Research frontiers

Cyclooxygenase-2 (COX-2) represents a key modulatory molecule in inflammation and carcinogenesis. COX-2 is known to have multiple tumorigenic effects. Increased expression of COX-2 has been observed in a variety of tumors including pancreatic cancer. In the literature, the prognostic significance of COX-2 expression including the role of antibody used for an evaluation of COX-2 expression profile have been discussed.

Innovations and breakthroughs

Recent reports have highlighted the role of COX-2 in carcinogenesis of pancreatic cancer and COX-2 has been suggested as a potential therapeutic target. This study confirmed the relationship of COX-2 to biological processes involved in pancreatic cancer, but its potential usefulness as a prognostic marker was not demonstrated.

Applications

The level of COX-2 expression does not seem to be a valuable independent prognostic factor, even if a significantly longer disease free survival and trends to longer overall survival were demonstrated in COX-2 negative cases using monoclonal antibodies. Immunohistochemical assessment of COX-2 expression is not superior to the conventional prognostic factors such as grade, stage and nodal status.

Terminology

COX-1 and COX-2 represent enzymes which are involved in the conversion of arachidonic acid to prostaglandins. COX-1 is expressed in many tissues under physiological conditions, while COX-2 is not constitutively expressed and several studies have suggested its positive role in regulation of growth and angiogenesis as well as inhibition of apoptosis in cancer.

Peer review

In this paper, Hermanova *et al* analyzed by immunohistochemistry COX-2 in pancreatic adenocarcinoma tissue samples. They concluded that the COX-2 expression does not seem to represent a valuable independent prognostic factor and is not superior to the conventional prognostic factors. Concerning the general view of the manuscript, it is characterized by a well done internal structure and acceptable quality of language. The data are clearly described and the statistical analysis seems to be correct.

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BRIEF ARTICLE

Non-small-bowel lesions encountered during double-balloon enteroscopy performed for obscure gastrointestinal bleeding

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Abstract

AIM: To report the incidence of non-small-bowel bleeding pathologies encountered during double-balloon enteroscopy (DBE) procedures and to analyse their significance.

METHODS: A retrospective study of a prospective DBE database conducted in a tertiary-referral center was conducted. A total of 179 patients with obscure gastrointestinal bleeding (OGIB) referred for DBE from June 2004 to November 2008 were analysed looking for the incidence of non-small-bowel lesions (NSBLs; all and newly diagnosed) encountered during DBE.

RESULTS: There were 228 (150 antegrade and 78 retrograde) DBE procedures performed in 179 patients. The mean number of DBE procedures was 1.27 per patient. The mean age (SD) of the patients was 62 ± 16 years old. There were 94 females (52.5%). The positive yield for a bleeding lesion was 65.9%. Of the 179 patients, 44 (24.6%) had NSBLs (19 of them had dual pathology with small-bowel lesions and NSBLs); 27 (15.1%) had lesions not detected by previous endoscopies. The most common type of missed lesions were vascular lesions.

CONCLUSION: A significant proportion of patients (24.6%) had lesions within reach of conventional endoscopy. Careful repeat examination with gastroscopy and colonoscopy might be required.

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Key words: Bleeding; Obscure gastrointestinal bleeding; Endoscopy; Double-balloon enteroscopy

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) accounts for approximately 5% of all patients with gastrointestinal (GI) bleeding[1], and in approximately 75% of these patients, lesions can be found in the small bowel[2-4].

Double balloon enteroscopy (DBE) was introduced by Hironori Yamamoto in 2001[5] and in the same year, the United States Food and Drug Administration approved the use of capsule endoscopy (CE)[6]. With the advent of CE and DBE, the management of OGIB has been revolutionised[7]. Mid-GI bleeding, previously considered almost inaccessible, is now effectively diagnosed and treated by a combination of CE and DBE as ambulatory procedures.

Often non-small-bowel lesions (NSBLs) are identified

during the search for pathology in the small bowel. This was a commonly reported phenomenon in push enteroscopy studies[8,9] and has been re-confirmed in CE studies, showing an incidence of NSBL of between 6.4%[10] and 38.8%[11]. The only DBE series looking at NSBL in OGIB reported a missed rate of 24.3%[12]; however, most of these patients had no prior CE. Our study investigated the incidence and clinical relevance of NSBL encountered during DBE performed for OGIB in patients who had prior CE and standard endoscopies.

MATERIALS AND METHODS

Study protocol

This is a single-center retrospective study in a tertiary referral teaching hospital in Sydney, Australia, conducted between June 2004 and November 2008. One endoscopist with experience in DBE and in therapeutic endoscopy performed the DBEs, with trainee registrars assisting with the outer tube. DBE was performed using the Fujinon enteroscope (Fujinon EN-450T5, Fujinon Corp., Saitama, Japan). DBE was performed *via* the antegrade (oral) or retrograde (anal) route, and the intention was to perform a targeted approach with the DBE. The approach was determined by the endoscopist, based on the time a lesion was seen in relation to the total small-bowel transit time on the CE study. If the lesion was within the proximal two thirds of the small bowel, then an antegrade DBE was used.

The DBE was performed with the patient either conscious or under deep sedation with a combination of intravenous midazolam (Pfizer, Bentley, Australia), fentanyl (Mayne Pharma Ltd., Mulgrave, Australia), and propofol (Fresofol 1%, Pharmatel Fresenius Kabi Pty Ltd, Hornby, Australia) administered by the assistant or attending anaesthetist. The preparation for the procedures included a fasting period of 8 h before the oral procedure and a routine sodium picosulphate-based bowel preparation (Picoprep, Pharmatel Fresenius Kabi Pty Ltd., Hornby, Australia), or sodium phosphate-based preparations (Fleet, Ferring Pharmaceuticals, Gordon, Australia) with a clear fluid diet the day before the procedure for the anal approach. The DBE technique was as previously described by the innovator H Yamamoto[5].

A failed retrograde DBE was defined as failure to insert the tip of the scope beyond the terminal ileum (approximately 20cm beyond the ileocaecal valve), as previously defined by Fry *et al*[12]. The antegrade DBE was considered to be a failure if the endoscopist failed to pass the duodeno-jejunal flexure.

Patients

All patients were referred by their specialist gastroenterologists or gastrointestinal surgeons to our tertiary referral service. All patients included in the study had OGIB as defined by the American Gastroenterological Association (AGA) criteria[13]. Thus, all patients had their initial investigations (EGD and colonoscopy) performed by their referring gastroenterologists within 6 mo of their CE. Information on patient demographics, previous investigations (endoscopic and radiological), findings and intervention with DBE, limitations of insertion, complication rates, and follow-up after therapy were all retrieved. Ethics board approval was obtained before data collection.

Patients were excluded if they had no prior CE, if the procedure could not be completed due to poor bowel preparation not allowing progress through the colon, procedures performed for colonic indications, sedation failure and technical/equipment failure.

NSBLs were defined as bleeding lesions proximal to the papilla of Vater or distal to the ileocecal valve (i.e. within reach of conventional upper and lower endoscopes). Small-bowel lesions (SBLs) were defined as bleeding lesions that lie between papilla of Vater and ileocaecal valve. Bleeding lesions were defined as lesions that definitely or probably explain the patient's bleeding or anaemia, such as active bleeding lesions, lesions with recent evidence of bleeding, or healed/healing lesions likely to have recently bled. Red marks and classical telangiectatic angioectasia were considered bleeding lesions whereas red spots were not.

Statistical analysis

The statistical software package SPSS for Windows Version 14 (SPSS Inc., Chicago, Ill) was used to analyse the data. mean ± SD was used to summarise data for continuous variables, whereas percentages were used for categorical variables.

RESULTS

Demographics

We identified 179 patients with OGIB who were referred for DBE. Two hundred and twenty eight DBE procedures (150 antegrade; 78 retrograde) were performed. Twenty seven patients had both antegrade and retrograde procedures. The mean number of DBE procedures was 1.27 per patient. The mean age (SD) of the patients was 62 ± 16 years. There were 94 females (52.5%).

Findings

DBE found a bleeding lesion in 118 (65.9%) patients and the distribution of bleeding lesions is summarised in Table 1. Ninety-three out of the total 179 patients (51.9%) had a positive finding localised to SBLs, with the most common being angioectasia (n = 64), followed by tumours/polyps (n = 13), and small bowel ulcers (n = 11) (Table 2). NSBLs were found in 44 (24.6%) patients (Table 3). Nineteen (10.6%) patients had dual bleeding pathologies in both the small bowel and outside the small bowel. Six (3.4%) were inconclusive due to failed procedures and all of them were of the retrograde group. Normal examinations were seen in 55 (30.7%) patients.

A total of 46 NSBLs were found in 44 patients. The majority of these 46 NSBLs were of vascular origin (n = 27), and the others were of peptic (n = 13) and neoplastic

Table 1 Findings by DBE performed for OGIB and anatomical distribution of bleeding lesions *n* (%)

Findings and distributions	Subjects
Positive for bleeding lesions	118 (65.9)
Small bowel only	74 (41.3)
Non-small bowel only	25 (14.0)
Small bowel and non-small bowel	19 (10.6)
Negative findings/normal	55 (30.7)
Inconclusive (failure of procedure)	6 (3.4)
Total	179 (100.0)

DBE: Double balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding.

Table 2 Small-bowel bleeding lesions found in the study cohort

Small-bowel lesions	*n*
Angioectasia	64
Tumours/polyps	13
Ulcers	11
Bleeding mucosa	2
Bleeding diverticula	2
Meckel's diverticulum	1
Stricture	1
Total	94[a]

[a]Ninety-four small bowel bleeding lesions were found in 93 patients. Nineteen patients also had NSBLs. NSBLs: Non-small-bowel lesions.

Table 3 Classification of NSBLs according to the nature and site of the lesions with totals for all and newly diagnosed lesions by DBE

Classification of NSBLs encountered on DBE	NSBL (*n*)	Newly diagnosed NSBL (*n*)
Vascular	27	16
Upper		
Stomach/duodenal angioectasia	12	8
GAVE	3	1
Oesophageal varices	2	1
Gastric varices	1	1
Lower		
Colonic angioectasia	6	4
Haemorrhoids	3	1
Peptic	13	9
Upper		
Hiatus hernia with Cameron's ulcer	5	3
Gastric ulcers/erosions	3	2
GERD (Los Angeles grade 3 or 4)	2	1
Haemorrhagic gastritis	1	1
Duodenal ulcers	1	1
Lower		
Colonic ulcer	1	1
Neoplastic	6	3
Upper		
Gastric polyps	4	1
Lower		
Colonic polyp	1	1
Colorectal carcinoma	1	1
Total	46[a]	28[b]

[a]46 NSBLs were found in 44 patients. Nineteen patients also had SBLs; [b]28 newly diagnosed NSBLs were found in 27 patients. GAVE: Gastric antral vascular ectasia; GERD: Gastro-esophageal reflux disease.

(n = 6) origins. Thirty-four of the lesions were found in the upper GIT; whereas 12 of the others were in the lower GIT. Of the 44 patients who had NSBL, 27 (15.1% from the total cohort) of their lesions were newly diagnosed during DBE, despite their prior investigations. These included angioectasia (n = 12), hiatus hernia with Cameron's erosions (n = 3), gastric ulcers (n = 2), and others (Table 3).

Interventions

Endoscopic interventions were performed in 96/118 (81.4%) patients who had positive bleeding lesions; 80 patients had therapeutic interventions (argon plasma coagulation in 75 and polypectomy in five), 14 patients had diagnostic biopsy, and two patients had tattoo placement for targeted surgery. Nineteen patients required medical treatment (18 were given proton pump inhibitors, one received chemotherapy for lymphoma), 11 patients were referred for surgery (eight neoplastic tumours, two bleeds that failed to be controlled endoscopically, and one Merkel's diverticulum).

Among the 44 patients who had NSBLs, endoscopic interventions were given in 23 patients; 20 of them had argon plasma coagulation and three had biopsies. Twenty one patients required medical treatments (18 required proton pump inhibitors and three were given treatment for variceal control). One patient had surgical resection for cecal carcinoma.

DISCUSSION

The AGA position paper concerning the evaluation and management of obscure GI bleeding was published in January 2000[14] and revised in 2007[13] following the availability of CE and DBE. The revised guidelines included CE as a pivotal investigation in OGIB. Depending of the findings on CE, DBE plays a key role in delivering targeted therapy or additional diagnostic clarification. One key element in the revised recommendations is for the inclusion of a repeat EGD and colonoscopy. The main point of contention is the timing of repeat procedures, as data is limited and hence the optimal timing of repeat procedures remains unclear.

Missed NSBLs have been a problem previously recorded in many published series. The literature for push enteroscopy has shown missed NSBL to account for up to 64.0% of all positive findings[8]. In another push enteroscopy series, missed upper GIT lesions accounted for 10.2% of 233 patients who had OGIB[9]. This issue has continued into the CE data, with several reports now showing missed NSBL in the order of 6.4%[10] to 38.8%[11]. Not surprisingly, Fry *et al*[12] in their DBE series reported the missed NSBL rate to be 24.3%; however, in that series CE was not available for all of their patients. In our cohort, NSBLs were detected in 24.6% of patients, a number strikingly similar to Fry *et al*[12]. However, all our patients had prior CE. In such circumstances, it stands to reason that CE would have identified some missed lesions and our figure should have been lower than that of Fry *et al*[12]. There was one clear explanation

for this. Not all NSBLs in our series were in fact completely missed. A significant number of these NSBLs were suspected or known by the referring doctors. However, referrals were made to exclude dual pathology (SBL and NSBL). Excluding those NSBLs that were known by previous investigations, the true missed rate for NSBL in our cohort was actually 15.1%.

There are several reasons to explain why lesions could be missed on conventional endoscopies. Lesions such as Dieulafoy's can be very difficult to diagnose as they can "resolve" by the time patients have their endoscopy. Often small-bowel bleeding can be caused by similar types of lesions and hence are dependent on the "timing" of DBE. This is best illustrated in study by Pennazio *et al*[15], in which the group of patients with ongoing overt bleeding had a 92.3% positive yield on CE when compared to groups with previous overt bleeding (12.9%) and guaiac-positive stool/iron deficiency anaemia (44.2%). If DBE could be performed during active bleeding then similar diagnostic rates with the addition of therapy should be achieved, limited only by the inferior total enteroscopy rates of DBE (77.5%[16] in the hands of experts).

Lesions such as Cameron's erosions also depend on the timing of endoscopy to confirm them as the bleeding source. Often, gastroenterologists referred such patients to our service with a high index of suspicion for Cameron's lesions but with no successful endoscopic timing. Hypovolemia and anaemia could cause lesions to look less obvious and this is particularly the case with small angioectasia, and could account for some missed lesions. Inadequate endoscopic examination also remains an issue. Despite the ease of EGD, careful examination for small lesions placed in awkward or in blind spots should be considered, and a more diligent and thorough examination is warranted in patients with OGIB. Likewise, an excellent colon preparation is required with a careful mucosal examination to exclude small mucosal bleeding points.

Missed lesions are highly relevant in the management of OGIB. Hartmann *et al*[17] studied 47 patients who had OGIB and were subjected to CE and intraoperative enteroscopy. Despite the accurate diagnosis from total small bowel examination and curative endoscopic and surgical treatments, on a mean follow up of 346 d, 25.5% of the patients had a re-bleed, and 19.1% of them required further interventions or blood transfusion. This suggests that despite the gold standard diagnostic and therapeutic approaches (in this case CE and IOE), many patients continue to bleed. Missed lesions could play a key role in these patients. In some series of patients with OGIB and a negative CE who continue to bleed, a repeat CE can find a lesion in up to 75% of patients[18]. Hence, patients who re-bleed need repeat examination of their entire GI tract to look for missed lesions.

In our cohort, 19 patients with NSBL had dual bleeding pathologies with both NSBL and SBL involvement. This suggests that if a significant NSBL is found during DBE, the small bowel examination should be completed, as approximately half of these patients will also have a significant small bowel finding. Conversely, if a definite bleeding site is found in the small bowel, then careful examination for NSBL is still warranted, even when no suspicion exists for NSBL due to the high true missed rate in our cohort of 15.1%.

The low diagnostic rates in our cohort reflect some real issues in managing patients with OGIB. Not all findings at CE are relevant and often the role of DBE is to exclude a lesion. One recurrent and classic situation is folds of bowel that appear like a polyp or a submucosal lesion. In addition, flecks of transported blood or mucous on the bowel surface could be mistaken as angioectasia or ulcers. Several studies, including our previous work[19], found that many CE findings are false positives.

We recognised the limitation of this study as being a retrospective series. It was difficult to classify all lesions into bleeding and non-bleeding lesions. We had to include a group of lesions as probable bleeders due to the size, appearance, history, and behaviour of the lesions. For instance, large polyps, extensive ulcerative disease, and malignant-looking lesions were considered probable bleeders, even though no blood or altered blood was detected on endoscopy. Likewise, diverticular disease might be considered a bleeder or non-bleeder based on their number and appearance.

In conclusion, NSBLs are a common finding during DBE, despite prior endoscopic examinations. Importantly, 15.1% of patients had unsuspected findings and hence careful examination of the entire GI tract is essential in all patients.

COMMENTS

Background

The problem of "missed" lesions seen at subsequent endoscopic examinations has been well documented in patients with obscure gastrointestinal bleeding (OGIB). The exact degree of this problem in the new era of capsule endoscopy and double-balloon enteroscopy remains unclear.

Research frontiers

The degree of the problem, the type of lesions missed, and the timing of repeat endoscopic examinations require further evaluation.

Innovations and breakthroughs

This paper shows that in a tertiary referral practice, the rate of missed lesions can be as high as 1 in 4 patients and thus highlights the relevance of repeat examinations in patients with ongoing OGIB.

Applications

In patients with ongoing OGIB without a definite finding on endoscopic evaluation, repeat procedures are worthwhile.

Terminology

OGIB is defined as bleeding with a negative endoscopy and colonoscopy.

Peer review

This paper is well written and provides new information on missed sites of bleeding within reach of conventional endoscopy and colonoscopy following capsule and regular endoscopy. The value of double balloon enteroscopy is also highlighted in diagnostic and therapeutic terms.

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BRIEF ARTICLE

Prognosis of cancer with branch duct type IPMN of the pancreas

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Abstract

AIM: To examine the coexistence of metachronous and synchronous cancer in branch duct intraductal papillary mucinous neoplasms of the pancreas (IPMN).

METHODS: We reviewed the records of 145 patients with branch duct IPMN between January 1991 and April 2008 and assessed the relationship between IPMN and intra- or extra-pancreatic carcinoma and the outcome of IPMN.

RESULTS: The mean observation period was 55.9 ± 45.3 mo. Among the 145 patients, the frequency of extra-pancreatic cancer was 29.0%. The frequency of gastric cancer, colon cancer, breast cancer, and pancreatic cancer were 25.5%, 15.7%, 13.7%, and 9.8%, respectively. Twenty (13.8%) of the patients died. The cause of death was extra-pancreatic carcinoma in 40%, pancreatic cancer in 25%, IPMN *per se* in 20%, and benign disease in 15% of the patients.

CONCLUSION: The prognosis for IPMN depends not on the IPMN *per se*, but on the presence of intra- or extra-pancreatic cancer.

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Key words: Intraductal papillary mucinous neoplasms of the pancreas; Long-term follow-up; Extra-pancreatic cancer; Pancreatic cancer; Prognosis

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INTRODUCTION

Intraductal papillary mucinous neoplasm of the pancreas (IPMN) shows a wide spectrum of histological presentations, ranging from adenoma with mild atypia to adenocarcinoma, and was first described by Ohashi *et al*[1] in 1980. IPMN is divided into two types, the main duct type and the branch duct type. In general, branch duct IPMN develops slowly and has a comparatively good prognosis. However, in several studies, it became evident that IPMN is a disease that very frequently coexists with cancer. Therefore, the prognosis of IPMN is more closely related to the coexisting disease than to IPMN

per se[2-8]. In the present study, the prognostic significance of the coexistence of metachronous and synchronous cancer in branch duct IPMN was investigated.

MATERIALS AND METHODS

The subjects were 145 patients with branch duct IPMN, including 33 resected and 112 non-resected cases, between January 1991 and April 2008, who had been periodically followed-up to date. The ratio of males to females was 79:66, the mean age was 66.3 ± 10.2 years (range: 29-89 years), and the mean observation period was 55.9 ± 45.3 mo (range: 1-217 mo). We classified IPMN into the main duct type without multi-locular dilatation of the branch duct, and the branch duct type. The Institutional Review Board of Tokyo Medical University reviewed and approved the entire study.

Indication of surgery for branch type IPMN

In our department, we consider that surgery for the branch type IPMN is indicated when the diameter of the cyst is 30 mm or more, the diameter of the mural nodule is 10 mm or more, and cytology findings of the pure pancreatic juice reveal suspected malignancy.

Strategy for branch type IPMN

For regular follow-up, we performed hematological tests, including those for tumor markers (carbohydrate antigen 19-9, and carcinoembryonic antigen) and pancreatic enzymes (amylase, lipase, and elastase-1), and several types of imaging (ultrasonography, endoscopic ultrasonography, computed tomography, or magnetic resonance cholangiopancreatography) every 6 to 12 mo.

Clinicopathological assessment of branch type IPMN

We assessed the clinicopathological findings as outlined below. (1) The relationship between IPMN and intra- or extra-pancreatic cancer; and (2) Prognosis of IPMN.

Statistical analysis

Normally distributed data are presented as mean ± SD. Significance was analyzed by the χ^2 test or Fisher's exact probability test, and Aspin-Welch's *t*-test. Statistically significant differences were denoted by $P < 0.05$. Statistical analysis was performed using Stat Mate Ⅲ (ATMS Co Ltd, Tokyo, Japan).

RESULTS

The resected patients had significantly greater mean cyst diameter, diameter of the main pancreatic duct, and height of node protrusion compared to those of non-resected patients ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively, Table 1).

The relationship between IPMN and intra or extra-pancreatic cancer

There was no statistically significant difference regarding the diameter of the cystic lesion, main pancreatic duct, and mural nodule between the branch type IPMN in patients with or without intra or extra-pancreatic cancer, regardless of whether the IPMN had been resected or not (Table 2).

Details of intra- or extra-pancreatic cancer in patients with IPMN are shown in Figure 1. Four patients (one man, three women) had metachronous double cancer. Gastrointestinal cancer accounted for 41.2% (21/51).

Incidence of, and detection time for, intra- or extra-pancreatic cancer in patients with IPMN are shown in Table 3. Briefly, detection prior to IPMN was seen in 56.9% (29/51), simultaneous detection of cancer and IPMN was seen in 27.5% (14/51), five of which were

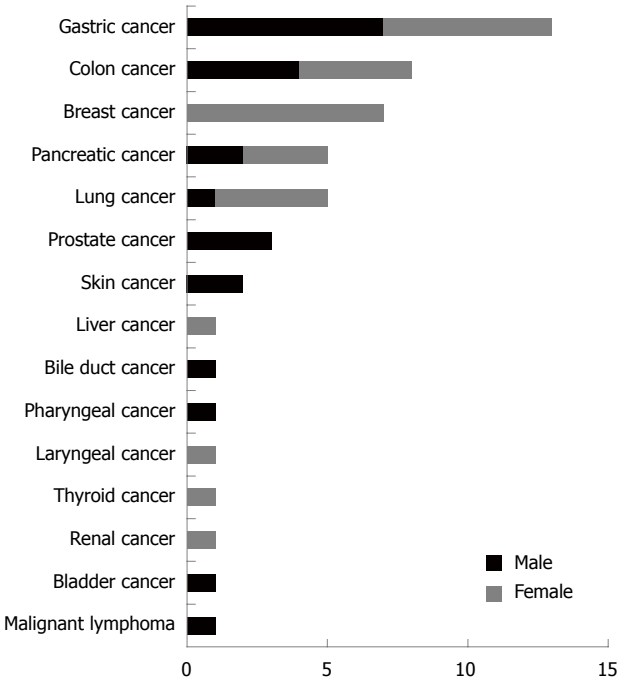

Figure 1 Details of intra- or extra-pancreatic cancers in patients with intraductal papillary mucinous neoplasms of the pancreas (IPMN).

Table 1 Characteristics of patients with branch duct type IPMN

	Total (n = 145)	Resection (n = 33)	Non-resection (n = 112)
Age (mean ± SD) (yr)	66.3 ± 10.2	63.9 ± 10.5	67.0 ± 10.1
		NS	
Male:female	79:66	20:13	59:53
		NS	
Median diameter of cystic lesion (mm)	20.1 ± 13.0	23.8 ± 11.5	19 ± 13.3
		$P < 0.05$	
Median diameter of MPD (mm)	2.2 ± 2.3	3.6 ± 3.3	1.7 ± 1.8
		$P < 0.01$	
Median diameter of mural nodule (mm)	1.6 ± 5.3	5.8 ± 9.8	0.3 ± 1.6
		$P < 0.01$	

P: *P* value (The presence of a statistically significant difference was denoted by $P < 0.05$). IPMN: Intraductal papillary mucinous neoplasms of the pancreas; SD: Standard deviation; MPD: Main pancreatic duct; NS: Not significant.

Table 2 Comparison of the branch type IPMN in patients with or without intra- or extra-pancreatic cancer

	IPMN with intra- or extra-pancreatic cancer (resection/non-resection)	IPMN without intra- or extra-pancreatic cancer (resection/non-resection)	*P* value
n	47 (16/31)	98 (19/79)	
Age	68.4 (65.0/70.0)	65.4 (63.2/66.4)	NS
Sex (male:female)	22:25 (7:9/15:16)	57:41 (13:6/44:35)	NS
Median diameter of cystic lesion (mm)	20.8 ± 11.8 (23.3 ± 10.4/19.5 ± 12.4)	19.7 ± 13.6 (23.0 ± 13.3/19.0 ± 13.7)	NS
Median diameter of MPD (mm)	2.2 ± 2.4 (3.5 ± 3.6/1.6 ± 1.2)	2.1 ± 2.3 (3.6 ± 2.9/1.7 ± 1.9)	NS
Median diameter of mural nodule (mm)	2.1 ± 5.7 (5.3 ± 8.7/0.5 ± 1.9)	1.3 ± 5.1 (5.6 ± 10.5/0.3 ± 1.5)	NS

Table 3 Incidence of, and detection time, for intra- or extra-pancreatic cancer in patients with IPMN (resected cases)

Neoplasm	Consciousness detection prior IPMN	Simultaneous with detection of carcinoma and IPMN	During follow-up for IPMN	Total
Gastric cancer	7 (1)	3 (3)	3 (0)	13 (4)
Colon cancer	4 (1)	4 (0)	0	8 (1)
Breast cancer	5 (2)	0	2 (0)	7 (2)
Pancreatic cancer	0	5 (4)	0	5 (4)
Lung cancer	3 (2)	1 (1)	1 (0)	5 (3)
Prostate cancer	2 (0)	0	1 (1)	3 (1)
Skin cancer	2 (1)	0	0	2 (1)
Liver cancer	1 (0)	0	0	1 (0)
Bile duct cancer	0	0	1 (0)	1 (0)
Pharyngeal cancer	1 (0)	0	0	1 (0)
Laryngeal cancer	1 (1)	0	0	1 (1)
Thyroid cancer	1 (0)	0	0	1 (0)
Renal cancer	0	1 (1)	0	1 (1)
Bladder cancer	1 (1)	0	0	1 (1)
Malignant lymphoma	1 (0)	0	0	1 (0)
Total	29 (9)	14 (9)	8 (1)	51 (19)

pancreatic cancer, and cancer was detected in 15.7% (8/51) during follow-up for IPMN.

Prognosis of IPMN, and clinicopathological findings in fatal cases of IPMN

Of the 145 patients with IPMN, 13.8% (20/145) died. As shown in Figure 2, the cause of death was extra-pancreatic cancer in 40% (8/20), pancreatic cancer in 25% (5/20), IPMN *per se* in 20% (4/20), and benign disease in 15% (3/20) of the patients. In particular, the cause of death of resected and non-resected cases is shown in Figure 2B and C.

The clinicopathological findings in four fatal cases of IPMN are shown in Table 4. The ratio of men to women was 3:1, the mean age was 64.5 years, two patients underwent surgery immediately after detection, and both had invasive adenocarcinoma accompanying IPMN. In one of the resected patients, the cyst diameter was 35.0 mm and the height of node protrusion was 19 mm. However, lymph node metastasis was observed post-operatively and the patient died of cancer 14 mo later. The other patient was found to have a nodular lesion with a diameter of 26.6 mm and pancreatic cancer was diagnosed. The patient underwent surgery, but hepatic metastasis occurred post-operatively and she died of cancer eight mo later. Of the

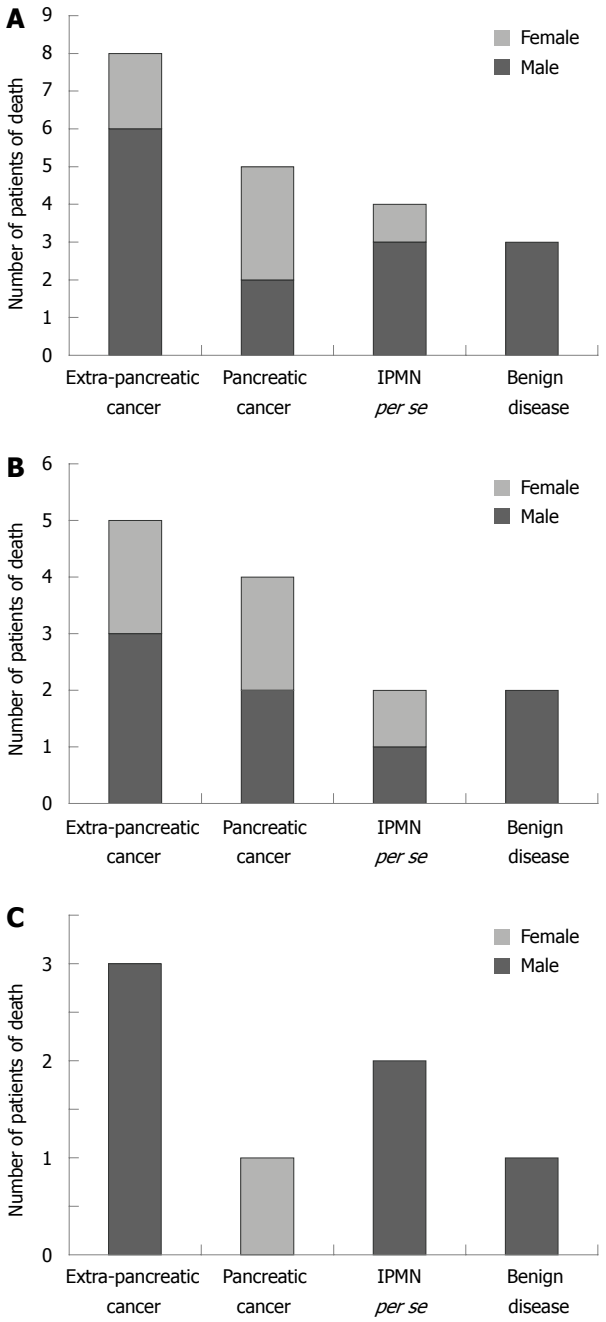

Figure 2 Number of patients of death. A: In patients with IPMN; B: In patients with resected IPMN; C: In patients with non-resected IPMN.

patients that died of cancer due to IPMN, two refused surgery and were therefore under observation only. They died

Table 4 Details of clinicopathological findings in fatal cases of IPMN

	Sex	Age (yr)	Region	Change of cystic lesion (mm)	Change of MPD (mm)	Change of mural nodule (mm)	Resection	Histopathological result	Period from diagnosis (operation) to death (mo)	Cause of death
Case 1	Male	89	Head	60→90	10→20	10→30	(-)	Adenocarcinoma (ch+, du+)	37	Cancer of death
Case 2	Male	58	Head	20→64	3→10	0→64	(-)	Adenocarcinoma (ch+, du+)	112	Cancer of death
Case 3	Male	53	Tail	35	1	19	(+)	Invasive ductal carcinoma derived from IPMN (INFα, mpd+)	15 (14)	Cancer of death
Case 4	Female	58	Head	0	1	26.6	(+)	Invasive ductal carcinoma derived from IPMN (INFβ, V2, ne1, du+, rp+)	9 (8)	Cancer of death

Table 5 Summary of the reports of concomitant extra-pancreatic cancers in patients with IPMN

Author (yr)	Number of IPMN patients	Extra-pancreatic cancers with IPMN patients	Age (mean ± SD)	Male: female	Follow-up period (mo)
Zhong (1996)	21	5	NA	NA	NA
Sugiyama (1999)	42	15	NA	NA	NA
Adsay (2002)	28	8	NA	NA	NA
Osanai (2003)	148	35	70.9	31:4	NA
Kamisawa (2005)	79	37	NA	NA	NA
Eguchi (2006)	69	32	NA	NA	NA
Choi (2006)	61	18	64.2 ± 8.4	13:5	65.8 ± 8.5
Yoon (2008)	210	77	65.3 ± 9.0	47:30	24.7
Ishida (2008)	61	15[1]	67.0 ± 7.0	12:3	50.9
Present study	145	42[2]	68.3 ± 9.0	20:26	49.8 ± 42.4
Total	864	284[3]			

[1]In those 2 patients who had metachronous double extra-pancreatic malignancies; [2]In those 4 patients who had metachronous double extra-pancreatic malignancies; [3]In those 6 patients who had metachronous double extra-pancreatic malignancies. NA: Not available.

at 37 and 112 mo, respectively, after detection. One non-resected patient initially had a 60 mm cyst and a 10 mm height mural nodule. When the patient died 37 mo later, the diameter of the cyst was 90 mm and the height of the mural nodule was 30 mm. The other non-resected patient initially had a cyst 20 mm in diameter with no mural nodule protrusion and when the patient died 112 mo later, the cyst had become a nodular lesion with a diameter of 64 mm.

DISCUSSION

We set out in determine the prognostic factors affecting cases of IPMN accompanied by cancer. In the present study, it became evident that cases complicated with metachronous and synchronous cancers in branch-type IPMN are quite common. Other studies that closely investigated such patients reported frequencies of extra-pancreatic cancer of 23.6%-46.8% and of a total of 864 IPMN patients, 32.9% (284/864) had extra-pancreatic cancer, which is almost the same as the subjects in the present study, where the frequency was 29.0% (42/145)[2-10] (Table 5). In addition, the most frequently occurring complications were gastric cancer and colon cancer, which was the same as in this study (Table 6).

On the other hand, other studies reported pancreatic cancer occurring in 3.8 to 9.2% of IPMN cases[6,11,12]. In our study it was 3.5% (5/145), which was slightly lower. In general, the etiology of second primary tumors is complex, involving multiple factors such as: (1) shared risk factors for the primary and secondary tumors, (2) host susceptibility to the development of specific tumors, (3) altered immunity as a result of the primary tumor and/or treatment, and (4) treatment with cytotoxic agents (radiotherapy and chemotherapy)[3]. In the present study, there was no clearly significant difference between cases complicated with other organ cancers and those without such complications in terms of cyst diameter, diameter of the main pancreatic duct, and presence or absence of mural nodules. This demonstrated that these data cannot be used as indices in the discovery of intra or extra-pancreatic cancer. Thus, further investigations, including follow-up studies, are necessary.

In the present study, most cases of IPMN were diagnosed after extra-pancreatic cancer was diagnosed. However, there are also reports stating that the time of IPMN diagnosis is unrelated to the onset of extra-pancreatic cancer[3,5].

The follow-up period and imaging modality for follow-up vary according to reports, and no consensus has been obtained. In this study, no case of pancreatic cancer occurred during follow-up. However, several researchers have reported pancreatic cancer after diagnosis of IPMN[6,11-15]. These findings demonstrate that periodic follow-up is necessary, even after IPMN is diagnosed.

The 5-year survival of patients with IPMN that undergo resection was reported to be for 36%-66%, even among those with invasive cancer. Thus, longer survival than that for pancreatic cancer can be expected[16-20]. According to our data, 25% of deaths were due to pancreatic cancer, while 40% were due to cancer of other organs. On the other hand, the follow-up periods of two patients who refused surgery and opted for conservative treatment, but who then died due to IPMN, were 37 and 112 m, which was relatively long. Our data demonstrated that to determine the prognosis for IPMN, intra- or extra-pancreatic cancer is the key factor, rather than the IPMN *per se*.

In conclusion, to decide on the prognosis for IPMN, the presence of intra- or extra-pancreatic cancer is the main prognostic factor, rather than IPMN *per se*. Therefore,

Table 6 Types of cancers reported concomitant with extra-pancreatic cancers in patients with IPMN

Neoplasm	Zhong (1996)	Sugiyama (1999)	Adsay (2002)	Osanai (2003)	Kamisawa (2005)	Egichi (2006)	Choi (2006)	Yoon (2008)	Ishida (2008)	Present study	Total
Gastric cancer	0	4	0	8	12	4	8	29	6	13	84
Colon cancer	3	5	0	11	7	8	4	16	4	8	66
Lung cancer	0	1	1	5	4	5	0	3	1	5	25
Bile duct cancer	0	1	0	4	1	NS	2	8	1	1	18
Prostate cancer	0	1	1	2	1	2	0	1	0	3	11
Renal cancer	0	1	1	2	0	2	0	3	1	1	11
Breast cancer	0	1	0	0	2	NS	0	0	0	7	10
Esophageal cancer	2	0	0	2	4	NS	0	0	1	0	9
Malignant lymphoma	0	0	0	0	1	NS	2	4	1	1	9
Bladder cancer	0	1	1	0	0	2	1	1	0	1	7
Liver cancer	0	0	0	1	2	NS	0	1	1	1	6
Thyroid cancer	0	0	0	0	0	NS	1	3	0	1	5
Ampullary cancer	0	0	1	0	0	NS	0	3	1	0	5
Skin cancer	0	0	1	0	0	NS	0	1	0	2	4
Uterine cancer	0	0	0	0	1	NS	0	2	0	0	3
Laryngeal cancer	0	0	0	0	1	NS	0	0	0	1	2
Pharyngeal cancer	0	0	0	0	1	NS	0	0	0	1	2
Ovarian cancer	0	0	0	0	0	NS	0	1	0	0	1
Duodenal cancer	0	0	0	0	0	NS	0	1	0	0	1
Other cancer or unknown	0	0	2	0	0	9	0	0	0	0	11
Total	5	15	8	35	37	32	18	77	17	46	290

there is no fixed algorithm regarding the follow-up period or methodology, but in all cases, close follow-up, especially for the development of cancer, is very important.

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COMMENTS

Background

Several investigators have suggested that the prognosis of the intraductal papillary mucinous neoplasms of the pancreas (IPMN) is more closely related to coexisting diseases than IPMN *per se*.

Research frontiers

In reports on studies that closely investigated such patients, the frequency of extra-pancreatic cancer was 23.6%-46.8% in a total of 864 IPMN patients, and 32.9% (284/864) had extra-pancreatic cancer, which corresponds well with the data from the subjects in the present study, where the frequency was 29.0% (42/145). On the other hand, many studies reported pancreatic cancer to occur in 3.8% to 9.2% of IPMN cases.

Applications

It appears to be important to determine the presence of intra- or extra-pancreatic cancer when examining the patients with IPMN.

Terminology

IPMN including branch duct type and main pancreatic duct type, have high risk for intra- or extra-pancreatic cancer, leading to death of patients.

Peer review

This is an interesting paper.

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BRIEF ARTICLE

Gender differences of low-dose aspirin-associated gastroduodenal ulcer in Japanese patients

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Abstract

AIM: To clarify the gender differences about the clinical features and risk factors of low-dose aspirin (LDA) (81-100 mg daily)-associated peptic ulcer in Japanese patients.

METHODS: There were 453 patients under treatment with LDA (298 males, 155 females) who underwent esophagogastroduodenoscopy at the Department of Gastroenterology and Hepatology of Hiratsuka City Hospital between January 2003 and December 2007. They had kept taking the LDA or started treatment during the study period and kept taking LDA during the whole period of observation. Of these, 119 patients (87 males, 32 females) were diagnosed as having LDA-associated peptic ulcer. We examined the clinical factors associated with LDA-associated peptic ulcer in both sexes.

RESULTS: A history of peptic ulcer was found to be the risk factor for LDA-associated peptic ulcer common to both sexes. In female patients, age greater than 70 years (prevalence ORs 8.441, 95% CI: 1.797-33.649, P = 0.0069) was found to be another significant risk factor, and the time to diagnosis as having LDA-associated peptic ulcer by endoscopy was significantly shorter than that in the male patients (P = 0.0050).

CONCLUSION: We demonstrated gender differences about the clinical features and risk factors of LDA-associated peptic ulcer. Special attention should be paid to aged female patients taking LDA.

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Key words: Low-dose aspirin; Gender; Peptic ulcer

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INTRODUCTION

Low-dose aspirin (LDA) is one of the main agents used for the prevention of thromboembolic vascular events, and has the advantages of both low cost and a prolonged duration of antiplatelet action[1]; however, it is associated with a doubling of the risk of gastrointestinal bleeding, even at doses as low as 75 mg daily[2,3]. The gender differences in the clinical manifestations of LDA-associated gastroduodenal mucosal injury have not been well studied. The aim of this study was to clarify the clinical features and risk factors of LDA-associated gastroduodenal mucosal injury in both sexes.

MATERIALS AND METHODS

Patients

There were 453 patients under treatment with LDA (298 males; median age, 71 years; age range, 43-93 years and 155 females; median age, 73 years; age range, 47-95 years) who underwent esophagogastroduodenoscopy (EGD) at the Department of Gastroenterology and Hepatology of Hiratsuka City Hospital between January 2003 and December 2007. The study was conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of Hiratsuka City Hospital. The exclusion criteria were: patients who were taking LDA for less than 7 d, patients who had been previously diagnosed or treated for LDA-associated gastroduodenal mucosal injury in the past, patients with gastric or duodenal cancer, history of gastric or esophageal surgery and any current active cancer. Detailed information about the drug treatment and other risk factors were obtained from the medical records and also from interviews with the patients when they underwent medical examination. Concomitant use of anti-ulcer drugs (proton-pump inhibitors (PPIs), Histamine type 2 receptor antagonists (H2RAs), muco-protective agents, anticoagulants and/or antiplatelets and antithrombotic agents was defined as starting with LDA or taken for more than 4 wk before receiving endoscopy.

Endoscopic assessments

Endoscopic examinations were performed in each patient. Only ulcers with whitish exudate that were over 5 mm in size[4] and had perceptible depth were included in the determination of the ulcer, with ulcer scars being excluded.

Helicobacter pylori infection status

The patients diagnosed to have ulcer were checked for *Helicobacter pylori* (*H. pylori*) infection using the *H. pylori* serum antibody assay, urea breath test, rapid urease test and *H. pylori* culture of two biopsy specimens obtained from the gastric body and antrum. If one of the tests was positive, the patient was labeled as *H. pylori*-positive, and if more than two of the tests were negative, the patient was labeled as *H. pylori*-negative.

Table 1 Clinical characteristics of patients taking LDA enrolled in the present study

	Patients with ulcer (n = 119)	Patients without ulcer (n = 334)	P-value
Gender (male/female)	87/32	211/123	NS
Age (yr), median (range)	73.0 (47-92)	72.0 (45-95)	NS
Male (n = 298)	70.0 (47-92)	71.0 (43-93)	NS
Female (n = 155)	77.0 (54-92)	73.0 (47-95)	0.0338
Underlying disease (male/female)			
Ischemic heart disease	77 (61/16)	205 (131/74)	NS
Cerebral infarction	22 (10/12)	70 (39/31)	NS
Atrial fibrillation	16 (13/3)	43 (28/15)	NS
Others	4 (3/1)	16 (13/3)	NS
Symptoms (male/female)			
Melena, hematemesis	46 (29/17)	1 (0/1)	< 0.0001
GI symptoms	31 (21/10)	107 (72/35)	NS[1]
Anemia	17 (14/3)	40 (21/19)	0.0156
Body weight loss	1 (1/0)	7 (5/2)	0.0087
Appetite loss	1 (1/0)	8 (2/6)	0.0028
None (medical check)	18 (15/3)	146 (96/50)	0.0025
Others	5 (2/0)	25 (15/10)	> 0.9999
Helicobacter pylori	62.3% (43/69)	-	
Male	66.7% (30/45)	-	
Female	54.2% (13/24)	-	

[1]Forty-six patients presented with melena and/or hematemesis and bleeding source in the stomach or duodenum identified by endoscopy and were excluded from GI symptoms in this analysis. LDA: Low-dose aspirin; GI: Gastrointestinal; NS: Not significant.

Statistical analysis

Statistical evaluation was carried out using Fisher's exact test, t-test or Mann-Whitney U-test and these variables were also evaluated by multivariate logistic regression model using a Wald statistic forward stepwise selection. The level of significance was set at $P < 0.05$. Statistical analyses were performed with Stat View software (SAS Institute, Cary, NC).

RESULTS

The baseline characteristics of the study population

The 453 patients under treatment with LDA who underwent EGD were included in this investigation. The baseline characteristics of the study population are summarized in Table 1. One hundred and nineteen (26.3%, 87 males, 32 females) of the 453 patients receiving LDA were diagnosed as having LDA-associated peptic ulcer, consisting of 92 patients with LDA-associated gastric ulcer (71 males, 21 females), 20 patients with duodenal ulcer (14 males, 6 females) and 7 patients with gastric and duodenal ulcers (2 males, 5 females).

The age of female patients with ulcer was greater than that of without ulcer (P = 0.0338). The most common indication for LDA was secondary prevention of ischemic heart disease (62.3%, 282/453). There were no significant differences in the distribution of underlying diseases between patients with ulcer and without ulcer (P = 0.8380). There were no significant differences in the presence of the gastrointestinal (GI) symptoms, defined as abdominal pain, nausea, reflux symptoms and/or the indigestion

Table 2 Univariate analysis to identify factors predictive of LDA-associated gastroduodenal ulcer in male and female patients

Factor	Male patients (n = 299)			Female patients (n = 155)		
	Patients with ulcer (n = 87)	Patients without ulcer (n = 211)	P-value	Patients with ulcer (n = 32)	Patients without ulcer (n = 123)	P-value
PPIs	1	22	0.0038	0	18	0.0253
H2RAs	16	53	NS	5	38	NS
Muco-protective agents	18	40	NS	6	18	NS
Hypertension	62	150	NS	21	88	NS
Hyperlipidemia	36	96	NS	14	51	NS
History of peptic ulcer	18	19	0.0109	9	9	0.0030
Diabetes mellitus	29	63	NS	11	30	NS
Insulin therapy	4	12	NS	3	3	NS
Anticoagulants	29	87	NS	11	35	NS
NSAIDs	3	25	NS	3	10	NS
Age ⩾ 70 yr	45	123	NS	28	85	0.0443
Alcohol	28	63	NS	6	12	NS
Smoking	22	36	NS	1	8	NS

PPIs: Proton-pump inhibitors; H2RAs: Histamine type 2 receptor antagonists; NSAIDs: Nonsteroidal anti-inflammatory drugs.

Table 3 Age-stratified prevalence of LDA-associated peptic ulcer in males and females

Age (yr)	Patients with ulcer (n = 119) (M/F)	Patients without ulcer (n = 334) (M/F)
40-49	2/0	4/3
50-59	15/2	20/7
60-69	24/2	64/28
70-79	33/17	94/55
80-89	10/11	26/27
90-	2/1	3/3

Table 4 Duration of LDA administration until diagnosis as having LDA-associated peptic ulcer in 106 patients

Month	Male (n = 78)	Female (n = 28)
-1	3	3
1-11	15	6
12-23	6	6
24-35	10	5
36-47	11	4
48-59	5	0
60-71	5	2
72-	23	2
Mean (min-max)	42 (1-223)	25.5 (1-130)

syndrome, between the patients with ulcer and without ulcer (P = 0.0761). Forty-six patients presented with melena and/or hematemesis and bleeding source in the stomach or duodenum identified by endoscopy were excluded from GI symptoms in this analysis. Moreover, even in patients with peptic ulcer, about half were asymptomatic.

Univariate analyses to identify predicted factors of LDA-associated gastroduodenal ulcer in both sexes were shown in Table 2. A history of peptic ulcer was found to be the only significant risk factor common to both sexes (male, P = 0.0109, female P = 0.0030), and age greater than 70 years was another risk factor in female patients (P = 0.0443). As shown in Table 3, there were no significant differences in the prevalence of LDA-associated peptic ulcer among age brackets in either sex (male, P = 0.3137, female P = 0.3612).

H. pylori infection status

The *H. pylori* infection status was determined in 69 patients (58.0%, 69/119), but remained unknown in the remaining 50 patients who were therefore not included in the statistical analysis of this parameter. Of the 69 patients, 62.3% (43/69) were *H. pylori*-positive [gastric ulcer, 69.1% (38/55); duodenal ulcer, 25.0% (2/8); gastric and duodenal ulcer, 50.0% (3/6)] and 26 (37.7%) were *H. pylori*-negative. There were no significant differences in the *H. pylori* infection rate between both sexes [male: 66.7% (30/45), female: 54.2% (13/24)] (P = 0.4344). The *H. pylori* infection rate was significantly lower in patients with LDA-associated ulcer as compared with that in patients with non-LDA-associated ulcer [including other nonsteroidal anti-inflammatory drugs (NSAIDs) associated ulcer] (77.0%, 419/544) diagnosed in the same study period (data are not shown in Tables) (P = 0.0112).

Time to diagnosis as having LDA-associated peptic ulcer by endoscopy

As shown in Table 4, we investigated the time from the start of LDA administration to the diagnosis as having LDA-associated peptic ulcer by endoscopy in 106 of the 119 patients (89.1%). LDA-associated peptic ulcer was diagnosed within the first month in 6 cases (5.7%) within the first 12 mo in 28 cases (26.4%), and within 5 years in 74 cases (69.8%). In female patients, the duration was significantly shorter than in male patients (P = 0.0050) (male: mean 42.0 mo; range, 1-223 mo, female: mean, 25.5 mo; range, 1-130 mo). The incidence of LDA-associated peptic ulcer seemed almost constant and did not decrease with time, at least over the follow-up period of 72 mo in this study.

Multivariate analysis to identify the risk factors of LDA-associated peptic ulcer in both sexes

Table 5 shows the results of multivariate analyses to identify the risk factors of LDA-associated peptic ulcer

Table 5 Multivariate analysis to identify risk factors of LDA-associated gastroduodenal ulcer in male and female patients

Factor	Odds ratio	95% CI	*P*-value
Male patients (n = 211)			
PPIs	0.069	0.009-0.555	0.0120
H2RAs	0.498	0.256-0.967	0.0396
History of peptic ulcer	3.745	1.734-8.088	0.0008
Female patients (n = 123)			
H2RAs	0.255	0.077-0.839	0.0245
History of peptic ulcer	6.439	1.874-22.131	0.0031
Age ≥ 70 yr	8.441	1.797-33.649	0.0069

Odds ratio: Prevalence odds ratio.

in both sexes. In male patients, a history of peptic ulcer (prevalence ORs 3.745, 95% CI: 1.734-8.088, P = 0.0008) was found to be associated with an increase in the risk, and a history of PPIs (prevalence ORs 0.069, 95% CI: 0.009-0.555, P = 0.0120) and/or H2RAs (prevalence ORs 0.498, 95% CI: 0.256-0.967, P = 0.0396) use was found to be associated with a decrease in the risk. In female patients, a history of peptic ulcer (prevalence ORs 6.439, 95% CI: 1.874-22.131, P = 0.0031) and age greater than 70 years (prevalence ORs 8.441, 95% CI: 1.797-33.649, P = 0.0069) were associated with an increase in the risk. Statistical analysis with respect to the use of PPIs could not be performed in female patients, because there was no female patient taking PPIs who was diagnosed as having LDA-associated peptic ulcer.

DISCUSSION

The present study demonstrated gender differences in the clinical features of LDA-associated gastroduodenal ulcer in Japanese patients. Previous reports have indicated age greater than 65 years, previous history of peptic ulcer[4,5], use of other NSAIDs[6] and *H. pylori* infection[7] as some of the risk factors of LDA-associated peptic ulcer, and the concomitant use of antisecretory drugs as a factor reducing the risk of LDA-associated peptic ulcer[5,8,9]. In this study, a history of peptic ulcer was found to be the risk factor of LDA-associated peptic ulcer common to both sexes. In female patients, advanced age or aging was found to be another significant factor. In elderly patients, gastric mucosal defenses are impaired because of the reduced mucus and bicarbonate secretion, and also the significant decrease in gastric mucosal perfusion. The reduced gastric mucosal defenses coupled with a reduced gastric prostaglandin biosynthesis may account for the higher susceptibility of the mucosa to damage-inducing agents[10]. In general, female subjects are at a relatively reduced risk of developing gastric ulcers[11]. In animal experimental models, female sex hormones have been demonstrated to show significant antiulcer activity and to promote healing of drug-associated ulcers (aspirin, indomethacin)[12,13]. Estrogen has also been reported to reduce the incidence of *H. pylori*-associated gastric mucosal injuries, despite having no effect on the *H. pylori* infection status. Female sex hormones may exert a protective effect against LDA-associated mucosal injury. In female patients of advanced age, the gradual decrease in the serum levels of female sex hormones after menopause and with advancing age, coupled with the reduced gastric mucosal defenses may be responsible for the increase in the risk of LDA-associated peptic ulcer.

In addition, time from the start of LDA until the diagnosis of LDA-associated peptic ulcer by endoscopy was shorter in the female patients. This gender difference, akin to the significantly higher relative risk and short time-to-onset of alcohol-related liver disease in women as compared to men for any given level of alcohol intake[14], is yet to be well elucidated. The incidence of peptic ulcer seemed almost constant and does not decrease with time, at least over the follow-up period of 72 mo in this study (Table 3). It has been suggested that NSAID-associated gastrointestinal bleeding occurs mainly within the first few weeks of treatment[15], which is a shorter period than that reported in LDA users. We should recognize that LDA-associated peptic ulcer may not occur for several years; therefore, it is important to continue antisecretory therapy, especially in high-risk patients. Future prospective cohort studies are needed to confirm this assumption.

COMMENTS

Background

The gender differences in the clinical manifestations of low-dose aspirin (LDA)-associated gastroduodenal mucosal injury have not been well-studied.

Research frontiers

The authors hypothesized that there were gender differences in clinical features of LDA-associated peptic ulcer, and it might be useful to identify the risk factors of LDA associated ulcer in both sexes.

Innovations and breakthroughs

In female patients, age greater than 70 years was found to be a significant risk factor of LDA associated peptic ulcer in addition to a history of peptic ulcer, and the time to diagnosis as having LDA-associated peptic ulcer by endoscopy was significantly shorter than that in the male patients.

Applications

By understanding the gender differences in clinical features of LDA-associated ulcer, this study may represent a future strategy for the prevention of LDA-associated peptic ulcer.

Terminology

Special attention should be paid to aged female patients taking LDA. People should also recognize that LDA-associated peptic ulcer may not occur for several years; therefore, it is important to continue antisecretory therapy, especially in high-risk patients.

Peer review

This study is adequately designed and performed. It means a contribution in a topic of high relevance.

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BRIEF ARTICLE

Chemotherapy with laparoscope-assisted continuous circulatory hyperthermic intraperitoneal perfusion for malignant ascites

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Abstract

AIM: To investigate the procedure, feasibility and effects of laparoscope-assisted continuous circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) in treatment of malignant ascites induced by peritoneal carcinomatosis from gastric cancers.

METHODS: From August 2006 to March 2008, the laparoscopic approach was used to perform CHIPC on 16 patients with malignant ascites induced by gastric cancer or postoperative intraperitoneal seeding. Each patient underwent CHIPC three times after laparoscope-assisted perfusion catheters placing. The first session was completed in operative room under general anesthesia, 5% glucose solution was selected as perfusion liquid, and 1500 mg 5-fluorouracil (5-FU) and 200 mg oxaliplatin were added in the perfusion solution. The second and third sessions were performed in intensive care unit, 0.9% sodium chloride solution was selected as perfusion liquid, and 1500 mg 5-FU was added in the perfusion solution alone. CHIPC was performed for 90 min at a velocity of 450-600 mL/min and an inflow temperature of 43 ± 0.2℃.

RESULTS: The intraoperative course was uneventful in all cases, and the mean operative period for laparoscope-assisted perfusion catheters placing was 80 min for each case. No postoperative deaths or complications related to laparoscope-assisted CHIPC occurred in this study. Clinically complete remission of ascites and related symptoms were achieved in 14 patients, and partial remission was achieved in 2 patients. During the follow-up, 13 patients died 2-9 mo after CHIPC, with a median survival time of 5 mo. Two patients with partial remission suffered from port site seeding and tumor metastasis,and died 2 and 3 mo after treatment. Three patients who are still alive today survived 4, 6 and 7 mo, respectively. The Karnofsky marks of patients (50-90) increased significantly ($P < 0.01$) and the general status improved after CHIPC. Thus satisfactory clinical efficacy has been achieved in these patients treated by laparoscopic CHIPC.

CONCLUSION: Laparoscope-assisted CHIPC is a safe, feasible and effective procedure in the treatment of debilitating malignant ascites induced by unresectable gastric cancers.

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Key words: Intraperitoneal hyperthermic perfusion; Laparoscopy; Chemotherapy; Gastric cancer; Malignant ascites

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INTRODUCTION

Malignant ascites are a common complication induced mainly by advanced ovarian cancer and also by gastrointestinal cancers (e.g. gastric cancer, colorectal cancer, and liver cancer). Once vast malignant ascites occur in patients with advanced gastric carcinoma or postoperative peritoneal seeding, disseminated seeding metastasis can be observed. Patients would lose the opportunity to undergo surgery, and nothing can be done except treating the pyloric obstruction or other complications. In addition, the prognosis is extremely poor and the survival time is generally as low as less than 2 mo[1].

In recent years, continuous circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) has appeared as a new auxiliary chemotherapy for abdominal malignant tumors. Preliminary clinical efficacy has been achieved in the prevention and treatment of malignant tumor metastasis[2-4].

Currently, laparoscope-assisted CHIPC has been used clinically to treat malignant ascites in a few medical institutions in the United States, France and Italy. In comparison with traditional CHIPC, laparoscope-assisted CHIPC has a good clinical profile and carries the benefits of definitive palliation of ascites with a minimal invasion, fewer traumas, less pain, shorter recovery time and more stable efficacy, thus avoiding the abdominal laparotomy and large incision necessary in patients with unresectable tumors[5-8]. However, little information related to the facilities, therapeutic temperature, velocity of drug perfusion or complications involved in laparoscope-assisted CHIPC has been available. This limits the widespread use of laparoscope-assisted CHIPC in clinical chemotherapy. Therefore, in this study, we present a detailed report on the use of laparoscope-assisted CHIPC in treatment of patients with malignant ascites induced by advanced gastric cancers.

MATERIALS AND METHODS

Inclusion criteria and clinical data

This protocol was approved by Cancer Hospital of Guangzhou Medical College. From August 2006 to March 2008, 16 patients with malignant ascites induced by advanced gastric cancers were treated *via* laparoscope-assisted CHIPC.

The diagnosis of primary gastric cancer complicated by malignant ascites was established and existence of peritoneal diffusive seeding was verified by laparoscopic examination. Thus these patients were not well suited for the tumor resection. They had no serious cardiopulmonary diseases. Informed consent of CHIPC and laparoscopic surgery was obtained from all the patients.

As shown in Table 1, of the 16 patients in this study, there were 7 men and 9 women aged 43-74 years (median age, 56 years). Karnofsky (KPS) marks ranged from 40 to 70 points and the course of disease ranged from 5-30 d (15 d on average). There were 7 primary cases of gastric cancer and 9 cases of postoperative gastric cancer, which were diagnosed by laparotomy, gastric fiberoptic endoscopy, serum tumor markers (CEA and CA199) and ascite cytology. Ultrasonic B and laparoscopic examinations displayed 4000-9000 mL seroperitoneum in all the patients. Five patients had obvious abdominal distention, abdominal pain and oliguria or anuria with high intraperitoneal pressure caused by ascites, and no apparent palliation was found after repeated abdominal puncture drainage, 11 patients had numerous free cancer cells within the ascites, 2 cases presented obvious bloody ascites, and 1 case presented chyle-like ascites.

Laparoscopic examination and chemotherapeutic catheter placement

After endotracheal anesthesia, a transverse cut (10 mm long) was performed at the belly, 5 mm below the umbilicus. The seroperitoneum was extracted as completely as possible; artificial pneumoperitoneum was established *via* an open procedure with a pressure of 13 mmHg (1 mmHg = 0.133 kPa); a 10-mm Trocar was inserted into the abdominal cavity *via* the working port. Thereafter, the laparoscope (10 mm and 30°) was inserted *via* the 10 mm Trocar to examine the abdominal viscera and tumors. The site, size and clinical stages of tumors were examined laparoscopically. Patients with peritoneal diffusive seeding and unresectable tumors were advised to receive laparoscope-assisted CHIPC.

In the process of laparoscope-assisted CHIPC, three new ports were prepared under the guidance of a laparoscope. On the right side, the second and third ports (both 10 mm long) were prepared at the cross-point of the mid-clavicular line and transverse surfaces, with two finger spaces above and below the umbilicus, respectively. On the left side, the fourth port (10 mm long) was prepared at the cross-point of the mid-clavicular line and transverse surface, with two finger spaces below the umbilicus. Thereafter, under the guidance of laparoscope, a 10 mm Trocar was inserted into the abdominal cavity *via* the working port. Two perfusion catheters were placed in the right superior abdominal cavity *via* the third and fourth working ports, respectively. One drainage catheter was placed in the Douglas' cavity of the lowest place in the pelvic cavity *via* the second working port. Then, the laparoscope was placed in the inferior abdomen and the Trocar was inserted. Subsequently, the laparoscope was pulled out, and the perfusion catheter was placed in the Douglas' cavity of the lowest place in the pelvic cavity under the guidance of the Trocar.

CHIPC procedures

CHIPC was performed by our self-developed "BR TRG

Table 1 Clinical data of patients undergoing laparoscope-assisted CHIPC in gastric cancer complicated by malignant ascites

Case No.	Gender	Age (yr)	Disease course (d)	Ascite volume (mL) [free cancer cells (+/-)]	Diagnosis	Examination	Pathology
1	F	46	17	7000 (+)	GC	GSB	MAC
2	M	58	21	4500 (+)	PGC	PP	M-LDAC
3	F	73	18	6000 (-)	GC	GSB	MDAC
4	F	58	12	4000 (+)	PGC	PP	MAC
5	M	76	7	4800 (+)	PGC	GSB	LDAC
6	M	58	9	9000 (+)	PGC	PP	SRCC
7	F	45	12	7000 (-)	GC	GSB	LDAC
8	F	59	80	4000 (+)	PGC	PP	SRCC
9	M	60	21	4500 (+)	PGC	PP	LDAC
10	M	67	13	4800 (-)	PGC	PP	M-LDAC
11	F	61	15	5000 (+)	GC	GSB	LDAC
12	M	52	14	6000 (+)	PGC	PP	MAC
13	F	68	9	5500 (+)	PGC	PP	LDAC
14	F	45	5	5800 (+)	GC	GSB	SRCC
15	M	59	12	6000 (-)	PGC	PP	LDAC
16	F	72	16	7200 (-)	PGC	GSB	M-LDAC

CHIPC: Circulatory hyperthermic intraperitoneal perfusion chemotherapy; GC: Gastric cancer; PGC: Postoperative gastric cancer; GSB: Gastroscopic biopsy; PP: Postoperative pathology; MAC: Mucinous adenocarcinoma; M-LDAC: Medium-low differentiated adenocarcinoma; SRCC: Signet ring cell cancer.

Ⅱ type high- precision hyperthermic perfusion intraperitoneal treatment system" (authorized by two patents from China: Intraperitoneal Hyperthermic Perfusion Treatment System with patent number ZL2006200613779 and Continuous Intraperitoneal Hyperthermic Perfusion Treatment Apparatus with patent number ZL2006200613764) with precise ± 0.2℃ temperature control, ± 5% flow control accuracy, and an automatic cooling function.

Patients completed the first session of CHIPC in the operating room under general anesthesia. The second and third sessions were performed in the intensive care unit on the first and second day after the operation. At the first session, 5-fluorine (1500 mg) and oxaliplatin (200 mg) were added into the 5% glucose solution (4500-6000 mL) as perfusion liquid. Then CHIPC was performed for 90 min with a velocity of 450-600 mL/min and an inflow temperature of 43 ± 0.2℃.

At the second and third sessions, pethidine hydrochloride (75 mg) and promethazine hydrochloride (25 mg) were administered by intramuscular injection less than 10 min before CHIPC. Propofol (3-8 mL/h, iv) as an anesthetic agent with a continuous vein pump. The dose was continuously adjusted according to patient status. The temperature and duration of CHIPC were the same as for the first session; 5-fluorine (1500 mg) was added to the 0.9% saline solution (4500-6000 mL) as perfusion liquid. When the third session of CHIPC was completed, the intra-abdominal perfusate and ascites were drained out. Two perfusion catheters were then pulled out, and a drainage catheter was remained most frequently in the body.

Evaluation and determination of efficacy

Two weeks later, malignant ascites were collected for the last time; the remained drainage catheter was withdrawn; and ultrasonic B reexamination was performed to evaluate ascite remission status. Thereafter, ultrasonic B or computed tomography (CT) was performed for follow-up, at least once a month. Ascite status and tumor progression were assessed. Karnofsky marks were used to evaluate ascite remission and patient quality of life before CHIPC and one and two weeks after the last CHIPC, respectively. All data were statistically analyzed using SPSS13.0 statistical software.

According to the modified WHO criteria of efficacy assessment in malignant tumors[1], clinical efficacy was divided into three grades: (1) complete remission (CR): ascites are completely absorbed after treatment sustained over 4 wk; (2) partial remission (PR): ascites are reduced by 50% and sustained over 4 wk; and (3) no consequence (NC): ascites reduced no obvious or increased after treatment.

RESULTS

Clinical efficacy

The intraoperative course was uneventful in all cases; mean operative period for laparoscope-assisted perfusion catheters placing was 80 min; no postoperative deaths or complications related to the laparoscope-assisted CHIPC procedure occurred in this study. After the first laparoscope-assisted CHIPC, the daily amount of ascite outflow was 100-300 mL. After the first day, ascite outflow gradually decreased. A week later, daily outflow was 0-30 mL. During the period from two weeks after laparoscope-assisted CHIPC to the end of follow-up, clinical CR of ascites and related symptoms were achieved in 14 of the 16 treated patients (90.5%), and PR was achieved in 2 patients (9.5%). Thus the objective remission rate (ORR = CR + PR) was 100%. The Karnofsky mark indicating patient quality of life was 50-90, which was increased significantly in comparison with before laparoscope-assisted CHIPC ($P < 0.01$).

Before laparoscope-assisted CHIPC, free cancer cells were found in the ascites of 11 patients (Figure 1A). However, free cancer cells degenerated and necrotized after the first CHIPC (Figure 1B). No intact free cancer cells were found in ascites after the second laparoscope-assisted CHIPC, but degenerated and necrotized cancer cells were

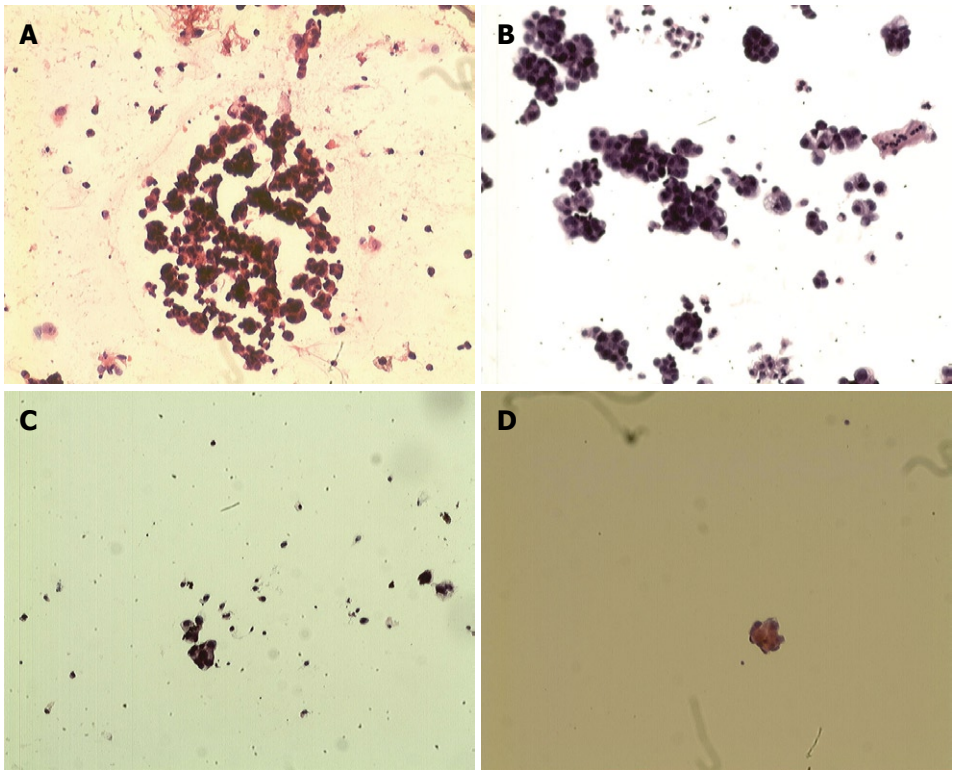

Figure 1 Cancer cells ascites. A: Numerous free cancer cells were found in ascites before circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) (HE, × 100); B: Intact free cancer cells in ascites clearly reduced in number, and some degenerated and necrotized after the first CHIPC (HE, × 100); C: No intact free cancer cells but rather degenerated and necrotized cells were found in ascites after the second CHIPC; D: No free cancer cells were found in ascites after the third CHIPC.

observed (Figure 1C). No intact free cancer cells were found in the abdomen after the third CHIPC (Figure 1D). In addition, bloody ascites in two cases and chyle-like ascites in 1 case turned clear after the first laparoscope-assisted CHIPC. In addition, the general status of patients improved after the third laparoscope-assisted CHIPC. Mental status, appetite and body weight improved, and symptoms of anemia were obviously alleviated. Thus, satisfactory initial clinical efficacy has been achieved in these patients treated by laparoscope-assisted CHIPC.

Side effects

In the course of laparoscope-assisted CHIPC, patients had no obviously abnormal vital signs except for transient fever, abdominal distension and bellyache. Dynamic re-examination in the anterior, median and posterior phases of laparoscope-assisted CHIPC revealed no anomalies in hepatorenal functions. Bone marrow suppression of Ⅰ-Ⅱ degrees was observed in three cases and slight gastrointestinal reaction in one case; and symptoms were remitted after symptomatic treatment. In addition, there were no complications, such as abdominal incision infection or adhesive ileus, after the laparoscope-assisted CHIPC operation.

Follow-up and prognosis

Clinical efficacies of laparoscope-assisted CHIPC in patients with gastric cancer complicated by malignant ascites are summarized in Table 2. All patients had complete follow-up data. During the follow-up (2-9 mo), 13 patients survived from 2 to 9 mo with a median survival time of 5 mo. The causes of death were intestinal obstruction (7 cases), gastrointestinal bleeding (3 cases), or cachexia (3 cases). Two patients with partial remission suffered from port-site seeding of the tumor and died within 2 and 3 mo after treatment, respectively. Three patients still alive today survived 4, 6 and 7 mo, respectively. Five patients failed in resection of the primary lesions in the re-laparotomy, and cecal fistula or ileal fistula was made in 4 patients for treatment of intestinal obstruction induced by tumor infiltration. The diagramed survival curves are shown in Figure 2.

DISCUSSION

Malignant ascites induced by malignant tumors in the abdominal cavity (e.g. ovarian cancer, gastric cancer, colorectal cancer, and other intra-abdominal cavity) are a common complication of intraperitoneal malignant tumors. The prognosis is extremely poor and the survival time is very short[1]. Gastric cancer is one of the most common intraperitoneal malignant tumors complicated by malignant ascites. Patients with gastric cancer complicated by malignant ascites suffer mostly from the rapid increase in ascites in addition to the clinical symptoms, vital signs and cachexia of primary diseases. However, diuretic treatment is not effective; single-puncture drainage of ascites leads to rapid re-growth; and multiple puncture drainage of ascites can cause severe complications such as hypoalbuminemia and hydrogen electrolyte disturbance. The ascites cause abdominal distention, abdominal pain and oliguria or anuria, which strongly impact the quality of life and render clinical treatment difficult. Therefore, suppressing or eliminating the rapid growth of ascites has great significance in the comprehensive treatment of patients with advanced gastric carcinoma or postoperative celiac diffusive seeding[1].

In recent years, CHIPC has appeared as a new auxiliary chemotherapy for intraperitoneal malignant tumors. Hyperthermic chemotherapeutic liquid at large doses fa-

Table 2 Clinical efficacy of laparoscope-assisted CHIPC in patients with gastric cancer complicated by malignant ascites

Case No.	Therapeutic outcome	Port-site seeding (+/-)	KPS mark		Survival time (mo)	Death/living
			Before treatment	After treatment		
1	CR	-	40	80	9	Death
2	CR	-	60	90	7	Death
3	CR	-	60	70	5	Death
4	CR	-	50	80	5	Death
5	CR	-	70	90	7	Death
6	CR	-	50	70	4	Death
7	PR	+	40	60	2	Death
8	CR	-	50	70	4	Death
9	CR	-	50	90	5	Death
10	CR	-	60	80	5	Death
11	CR	-	60	80	4	Death
12	PR	+	50	70	3	Death
13	CR	-	40	80	7	Death
14	CR	-	70	80	7	Living
15	CR	-	60	70	4	Living
16	CR	-	60	80	6	Living

KPS: Karnofsky; CR: Complete remission; PR: Partial remission.

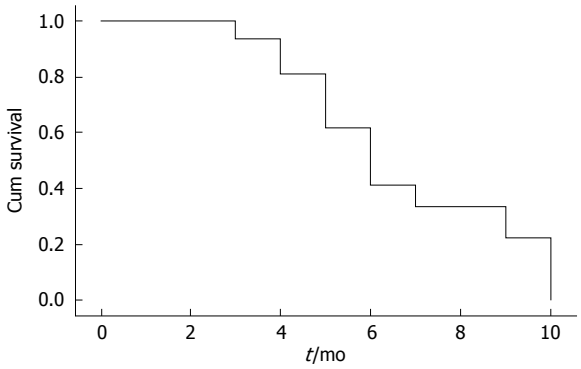

Figure 2 Diagrammed survival curves of patients undergoing laparoscopic CHIPC in gastric cancer complicated by malignant ascites.

cilitates a full and effective reach of the drugs to the tiny celiac carcinoma metastases. Perfusion chemotherapy affects mechanical removal of intra-abdominal free cancer cells. During the perfusion, chemotherapeutic drugs are locally administered to the abdominal cavity, which leads to a high, constant and sustained local drug concentration. However, fewer drugs enter into the systemic circulation, which reduces the systemic side-effects. Therefore, CHIPC has obvious advantages in comparison with simple intraperitoneal chemotherapy in the treatment of malignant ascites. Preliminary efficacy has been achieved in the prevention and treatment of the peritoneal seeding of malignant tumors[5-8].

In the process of laparoscope-assisted CHIPC, the site, size and clinical stages of tumors are laparoscopically examined. Patients with peritoneal diffusive seeding and unresectable tumors are advised to undergo laparoscope-assisted CHIPC. Laparoscope-assisted CHIPC integrates the benefits of a definitive palliation of ascites with a minimal invasion, which leads to fewer traumas, less pain, shorter recovery time and solid efficacy, thus avoiding the abdominal laparotomy and large incision necessary in patients with unresectable tumors. Therefore, laparoscope-assisted CHIPC is a potential therapy for malignant ascites[9-12].

Ferron *et al*[13] performed laparoscope-assisted CHIPC on five adult pigs in France. Trocar was placed in the four celiac corners; omental total resection was performed. The final Trocar below the umbilicus was positioned using Dapdics, which is helpful in operating upon the ansa intestinalis. The abdominal cavity was filled with hot saline for 30 min at 43℃. As a result, the liquid was well distributed in the intra-abdomen with the appropriate temperature, and no surgical complications occurred. Therefore, the authors suggest that laparoscope-assisted CHIPC is safe and feasible for chemotherapy of malignant ascites.

Gesson-Paute *et al*[14] performed laparoscope-assisted CHIPC on ten adult pigs to investigate the changes in pharmacokinetics. In the experiment, perfusion and drainage catheters were laparoscopically placed in the upper and lower abdominal quadrants. In control animals, drainage catheters were placed laparotomically. CHIPC was performed with perfusion liquid containing oxaliplatin (460 mg/m^2) for 30 min at 41-43℃. The perfusion liquid was sampled every minute to determine the drug concentration. The results showed that the operation was performed smoothly without complications. Drug absorption rate was 41.5% in the experimental group but 33.4% in the control. This difference was not statistically significant (P = 0.0543, n = 5). The half-life of oxaliplatin in laparoscope-assisted CHIPC was significantly shorter than that in laparotomic CHIPC (37.5 min *vs* 59.3 min, P = 0.02), which indicates that the adsorption of oxaliplatin in laparotomic CHIPC is significantly faster than that in laparotomic CHIPC, leading to increased clinical efficacy. In addition, the celiac drug kinetic curvature for laparoscopic and laparotomic CHIPC was 16.3 *vs* 28.1, P = 0.02, respectively, which reflects intraperitoneal barrier penetration of oxaliplatin. Therefore, the authors believed that laparoscope-assisted CHIPC-associated pharmacokinetics elucidate intraperitoneal drug adsorption and support the viewpoint that laparoscope-assisted CHIPC is safe, with robust clinical efficacy.

Facchiano *et al*[15] treated five patients with malignant ascites after palliative resection of gastric cancer by laparoscope-assisted CHIPC in a hospital affiliated to the Paris University. Celiac perfusion and drainage catheters were laparoscopically placed in the upper and lower abdominal quadrants, and laparoscope-assisted CHIPC was performed with perfusion liquid containing mitomycin plus platinum for 60-90 min at an inflow temperature of 45℃. The results showed that the operation went on smoothly without related complications for a mean 181 min; malignant ascites were eliminated in all 5 cases. The authors suggest that laparoscope-assisted CHIPC in patients with malignant ascites after palliative resection of gastric cancer is safe and feasible, with robust clinical effect.

Garofalo *et al*[16] performed laparoscope-assisted CHIPC on 14 cases of malignant ascites and five cases of gastric cancer, three cases of colorectal cancer, three cases of ovarian cancer, two cases of breast cancer and one case of intraperitoneal pseudomyxoma, respectively. Laparoscope-assisted CHIPC was performed with chemotherapeutic drugs (e.g. carboplatin, cisplatin and mitomycin according to the primary tumor type) for 90 min at inflow temperature of 42℃. Four drainage catheters were laparoscopically placed in the upper and lower abdominal quadrants and were pulled out after perfusion. The results indicated that malignant ascites had been well controlled in all 14 cases, and postoperative CT scanning confirmed pelvic effusion in one case. The authors conclude that laparoscope-assisted CHIPC is safe and efficient for chemotherapy of patients who suffer from advanced malignant tumors complicated by ascites and peritoneal seeding and who are not suited for cytoreductive surgery. However, the prerequisites are surgeon's experience with laparoscopic tumor staging and patient's capacity to undergo laparoscope-assisted CHIPC.

Knutsen *et al*[17] performed laparoscope-assisted CHIPC on five patients who underwent laparotomic cytoreductive surgery three to eight weeks earlier. The primary diseases included adenocarcinoma of the cecum in one case, appendiceal adenocarcinoma in three cases and gallbladder adenocarcinoma in one case. The inflow temperature was 42.1℃ and outflow temperature was 40.5℃. After the operation, one patient exhibited port-site seeding, one patient died of tumor progression, and three patients survived without tumor progression and enjoyed a normal quality of life within four months after laparoscope-assisted CHIPC. The author suggests that laparoscope-assisted CHIPC after laparotomic cytoreductive surgery can guarantee the constant velocity of perfusion and maintain a stable intra-abdominal temperature.

In the process of CHIPC, drainage tubes tend to be plugged up by the great omentum or intestinal canals, so unobstructed drainage is the key to successful CHIPC. Through the long-term observation of clinical practice, we have mastered reliable means to place laparoscope-assisted CHIPC tubes and prevent jamming of the drainage tube. Drainage tubes which pass through the perforations of the right upper quadrant or left upper quadrant are placed in the lowest pelvic cavity (Douglas' cavity), and perfusion tubes are placed in the abdominal hepatorenal recess and the splenic recess through the perforations of the right lower quadrant or left lower quadrant, respectively. When undergoing laparoscope-assisted CHIPC, the patient is placed in the supine or head-high-foot-low (15º) positions. After the abdominal cavity is full of liquid, drainage tubes are open to drain the liquid, with help of the body position. The great omentum and intestinal canals are difficult to position within the pelvic cavity and therefore unlikely to plug up the drainage tube, thus unobstructed circulatory perfusion is needed. If pelvic drainage tubes are plugged up, smooth drainage can be ensured by changing infusion tube from the right upper quadrant (hepatorenal recess) into drainage tube to the lower quadrant (Douglas' cavity) and changing the patient's position from head low to supine position. In this study, all the patients suffered from gastric cancer-induced malignant ascites and their great omentums were all excised or contracted into clumps. The abdominal cavity volume was big enough to ensure smooth drainage; there was no failure in the drainage tube jam in laparoscope-assisted CHIPC.

We have successfully developed a High Precision Intraperitoneal Hyperthermic Perfusion Treatment system with independent intellectual property rights. In this study, we used this machine for laparoscope-assisted CHIPC to treat 16 patients with malignant ascites induced by peritoneal carcinomatosis from gastric cancers.All patients with ascites have remitted which indicates a 100% effective rate as reported by other authors[15,17]. Bloody ascites in two cases and chyle-like malignant ascites in one case turned clear very quickly after the first laparoscope-assisted CHIPC. The median survival time was 5 mo, which is prolonged as compared with the traditional therapy, and other reports as well[16,17]. General status, mental status, appetite and body weight improved, symptoms of anemia were alleviated, and initial clinical efficacy was satisfactory in the patients. All these results imply that laparoscope-assisted CHIPC has good clinical efficacy.

In addition, our clinical observations indicate that the primary tumors remain or even that peritoneal seeding foci are aggravated after laparoscope-assisted CHIPC. However, the symptoms associated with malignant ascites decreased or disappeared completely and ultrasonic B examination has not revealed ascite recurrence. The theory of this system remains to be elucidated. At the same time, bloody and chylous ascites became clear very quickly after laparoscope-assisted CHIPC, a process which also requires further studies.

Port-site seeding is a frequent problem for laparoscopic doctors. Patients who undergo laparoscope-assisted CHIPC have many free cancer cells in the abdominal cavity and more severe port-site seeding. Two patients with port-site seeding are included in this study and exhibited only partial remission after laparoscope-assisted CHIPC, which indicates poor clinical efficacy. However, this study includes only a limited number of cases, and the influence of laparoscope-assisted CHIPC on the quality of life and long-term survival of patients requires further researches.

In conclusion, laparoscope-assisted continuous circulatory intraperitoneal hyperthermic perfusion chemotherapy

(CHIPC) in gastric cancer complicated by malignant ascites guarantees the uniform velocity of perfusion liquid, maintains the stable intra-abdominal temperature, facilitates a full and effective reach of the drugs to the celiac cancers, and has advantages such as minimal invasion, less pain and short recovery time. These factors contribute to achieving a good clinical efficacy, including improved quality of life and prolonged survival. Therefore, laparoscope-assisted CHIPC is safe and feasible for a widespread clinical use.

COMMENTS

Background

Malignant ascites is a common complication of advanced peritoneal malignant tumors, and treatment of this malady is very difficult clinically. Continuous circulatory hyperthermic intraperitoneal perfusion chemotherapy (CHIPC) has achieved satisfactory therapeutic effects in treatment of malignant ascites.

Research frontiers

Laparoscope-assisted CHIPC integrates the benefits of definitive palliation of gastric cancer complicated by malignant ascites with fewer traumas, less pain, shorter recovery time and more stable efficacy, thus avoiding the abdominal laparotomy and large incision necessary in patients with unrespectable tumors.

Innovations and breakthroughs

Laparoscope-assisted CHIPC is a safe, feasible and effective procedure to treat debilitating malignant ascites induced by unresectable intraperitoneal carcinomatosis of gastric cancers.

Applications

Laparoscope-assisted CHIPC for gastric cancer complicated by malignant ascites has advantages such as minimal invasion, less pain and short recovery time. These factors contribute to achieving a good clinical efficacy, including improved quality of life and prolonged survival.

Terminology

CHIPC is a novel adjuvant therapy for intra-abdominal malignancies. High-doses of warm chemotherapy liquid are administered intra-abdominally so that chemotherapy drugs can have a full contact with the tumor metastases. Chemotherapeutic agents administered into the abdominal cavity have a higher, constant, and persistent drug concentration but less drugs entering the circulatory system, and few systemic toxic side effects.

Peer review

The scientific and innovative contents as well as readability of this paper can reflect the advanced levels of the clinical research in gastroenterology both at home and abroad.

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BRIEF ARTICLE

Remission induction and maintenance effect of probiotics on ulcerative colitis: A meta-analysis

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Abstract

AIM: To evaluate the induction of remission and maintenance effects of probiotics for ulcerative colitis.

METHODS: Information was retrieved from MEDLINE, EMBASE, and the Cochrane Controlled Trials Register. The induction of remission and promotion of maintenance were compared between probiotics treatment and non-probiotics treatment in ulcerative colitis.

RESULTS: Thirteen randomized controlled studies met the selection criteria. Seven studies evaluated the remission rate, and eight studies estimated the recurrence rate; two studies evaluated both remission and recurrence rates. Compared with the non-probiotics group, the remission rate for ulcerative colitis patients who received probiotics was 1.35 (95% CI: 0.98-1.85). Compared with the placebo group, the remission rate of ulcerative colitis who received probiotics was 2.00 (95% CI: 1.35-2.96). During the course of treatment, in patients who received probiotics for less than 12 mo compared with the group treated by non-probiotics, the remission rate of ulcerative colitis was 1.36 (95% CI: 1.07-1.73). Compared with the non-probiotics group, the recurrence rate of ulcerative colitis patients who received probiotics was 0.69 (95% CI: 2.47-1.01). In the mild to moderate group who received probiotics, compared to the group who did not receive probiotics, the recurrence rate was 0.25 (95% CI: 0.12-0.51). The group who received *Bifidobacterium bifidum* treatment had a recurrence rate of 0.25 (95% CI: 0.12-0.50) compared with the non-probiotics group.

CONCLUSION: Probiotic treatment was more effective than placebo in maintaining remission in ulcerative colitis.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Probiotics; Meta-analysis

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INTRODUCTION

Inflammatory bowel disease is a chronic recurrent disease, which mainly consists of ulcerative colitis and Crohn's disease, and whose causes are as yet unclear. It is still assumed that inflammatory bowel disease is caused by integrative factors, such as genetic susceptibility, an imbalance between gastrointestinal probiotics and pathogens, an impairment of intestinal epithelial cells, and

host immune dysfunctions. Many clinical and research studies have indicated that intestinal flora dysbacteriosis contributes to the pathogenesis of ulcerative colitis. Probiotics are non-pathogenic beneficial flora, which have important effects on maintaining the balance of intestinal flora[1]. Probiotics can adjust the metabolic activity of the intestinal flora and their components by preventing bacterial overgrowth and by maintaining the integrity of the intestinal mucosal barrier, thereby adjusting and stabilizing the intestinal environment[2,3].

Inducing remission and preventing recurrence and complications are the primary goals of inflammatory bowel disease treatment[4]. Many ulcerative colitis patients experience a short period of remission after induction but then must be treated by surgery after recurrent attacks. Postoperative pouchitis is always caused by the reduction of *Lactobacillus lactis* and *Bifidobacterium*. Many studies have discussed the positive effects of probiotics for treating stomach and intestine diseases, including ulcerative colitis[5-7]. However, the sample size has been relatively small, such that there is no definitive evidence as to whether probiotics are helpful. Thus, this paper systematically evaluates probiotics' curative effects for treating ulcerative colitis based on existing random control trials.

MATERIALS AND METHODS

Retrieval strategy

Data was retrieved from the databases: We searched the data from MEDLINE (1966 to August 2009), EMBASE (1980 to August 2009), and the Cochrane Controlled Trials Register (1995 to August 2009). The keywords were used below: probiotics, inflammatory bowel disease, ulcerative colitis, *Escherichia coli*, *Lactobacillus*, *Bifidobacterium,* and yeasts. Language was limited to studies published in English. Moreover, abstracts presented at main international gastrointestinal disease meetings (including Digestive Disease Week of the American Gastroenterological Association, the World Congress of Gastroenterology) for the past five years were analyzed by joint manual retrieval. The retrieval results were reviewed by two evaluators, and a third person was consulted if the two evaluators' opinions were different.

Selection criteria: All the random control experiments that compared probiotics with ulcerative colitis treatment medicine or placebo were collected, including abstracts and full texts. The selection criteria were as follows: (1) adult and pediatric studies were included; (2) the experiments compared the curative effect of probiotics with standard therapy for ulcerative colitis or placebo; (3) all were random control tests; (4) abstracts and meeting presentations were selected; and (5) patients had a definite diagnosis of ulcerative colitis using definite diagnosis standards. Reviews and case reports were excluded.

Data retrieval and quality assessment

Two researchers selected the papers after reading the titles, abstracts, and full texts. They evaluated the quality of each selected paper and retrieved the data. If they had different opinions, a third researcher helped them to determine the applicability of the paper in question. The quality of the methods used by the selected random control experiments was assessed by the Cochrane Reviewer Handbook 5.0 random control experiment quality assessment standard. The following methods were evaluated: (1) Random method: random method right, random method not described, random method error; (2) Allocation concealment: concealment method right, concealment method not described, concealment method error, concealment method not used; (3) Blind method: whether the evaluator was blinded to the conditions of the experiment; and (4) Whether lost or exit: if there are lost or exit conditions, the reasons are indicated clearly or not, and whether intention-to-treat analysis was used. According to these four quality criteria, all answers that were "right or enough", where the possibilities for bias are the least; the assessment was level "A". If one or more criteria were not described, it means that the assessment was partially satisfied. In this condition, there is a possibility of relative bias, and the assessment was designated as level "B". If one or more criteria were erroneous or not used, there was a high possibility of relative bias, and the assessment was designated as level "C".

Statistical analysis

The relative risk and 95% confidence interval (95% CI) was calculated based on the data. Statistical analysis was performed with Cochrane Collaboration's Revman 5.0. Heterogeneity analyses were performed on the experimental results. If there was obvious heterogeneity, the random effects model was chosen, if not, the fixed effects model was chosen.

Whether there was publication bias could be observed by an inverted funnel plot, and the bias level was assessed by the formula:

$$\sum_{j-1}^{m} \ln(O\hat{R}_j) = 0$$

$$m = [\frac{k \ln(O\hat{R})}{1.96}]^2 \bar{\omega} / k - k$$

Where k is the number of selected papers; m is the least unpublished number of reports that yield a combined effect size with no statistical significance; $\bar{\omega}$ is the average weight of k published reports (reciprocal of variance); and $\ln(O\hat{R})$ is the estimate value logarithm of the combined effect size.

RESULTS

A total of 286 papers were retrieved. After a thorough screening of the titles, abstracts, or full texts and excluding reviews, uncontrolled tests, and basic research, 13 papers were ultimately selected[5-11] (Figure 1). One of the 13 papers was published as an abstract, 12 of were published as full text; seven of them evaluated the remission rate, eight papers assessed the recurrence rate, and two papers evaluated both the remission rate and recurrence rate. The span of the research or the duration of

Table 1 Characteristics of included studies

Study	Disease severity	Probiotic	Control group	Dose of pro-biotic/ day)	Treatment duration	*N* (probiotic/ control group)	induction or maintenance of remission *N* (probiotic/ control group)
Miele *et al*[13], 2009	Mild-to-moderate	VSL#3	Placebo	450-1800 × 10^9	12 mo	14/15	Induction of remission 13/4; Maintenance of remission 11/4
Sood *et al*[12], 2009	Mild-to-moderate	VSL#3	Placebo	3.6 × 10^{12}	12 wk	77/70	Induction of remission 33/11
Henker *et al*[15], 2008	Inactive UC	*E. coli* Nissle 1917	5-ASA	5 × 10^{10}	12 mo	24/10	Maintenance of remission 18/7
Zocco *et al*[20], 2006	Inactive UC	Lactobacillus GG	Mesalazine	18 × 10^9	12 mo	65/60/62	Maintenance of remission 55/48/52
Matthes *et al*[11], 2006	Mild-to-moderate	*E. coli* Nissle 1917	Placebo	10-40 × 10^8	4 wk	46/11	Induction of remission 20/2
Furrie *et al*[9], 2005	Active	Synbiotic (*Bifidobacterium longum* + inulin-oligofructose)	Potato starch and sachet of 6 g powdered maltodextrose	4 × 10^{11}	4 wk	9/9	Induction of remission 5/3
Tursi *et al*[8], 2004	Mild-to-moderate	Balsalazide/VSL#3	Mesalazine/ balsalazide	900 × 10^8	8 wk	30/30/30	Induction of remission 24/21/16
Kruis *et al*[18], 2004	Inactive	*E. coli* Nissle 1917	Mesalazine	2.5-25 × 10^9	12 mo	162/165	Maintenance of remission 89/104
Kato *et al*[10], 2004	Mild-to-moderate	*Bifidobacterium breve* strain Yakult *Bifidobacterium bifidum* strain Yakult *Lactobacillus acidophilus*	BFM without *B. bifidum* and *L. acidophilus*	10^9	12 wk	10/10	Induction of remission 4/3
Cui *et al*[16], 2004	Active	*Bifidobacteria*	Starch	1.26 g/d	8 wk	15/15	Maintenance of remission 12/1
Ishikawa *et al*[17], 2003	Mild-to-moderate	*Bifidobacterium Breve* *Bifidobacterium Bifidum* *Lactobacillus acidophilus* YIT 0168	BFM without these *Bifidobacteria*	10 × 10^8	12 mo	11/10	Maintenance of remission 8/1
Rembacken *et al*[14], 1999	Active	*E. coli* Nissle 1917 serotype O6: K5: H1	Mesalazine	5 × 10^{10}	12 mo	57/59	Induction of remission 39/44; Maintenance of remission 31/27
Kruis *et al*[19], 1997	Inactive UC	*E. coli* Nissle 1917 serotype O6: K5: H1	Mesalazine	50 × 10^9	12 wk	50/53	Maintenance of remission 42/51

E. coli: *Escherichia coli*.

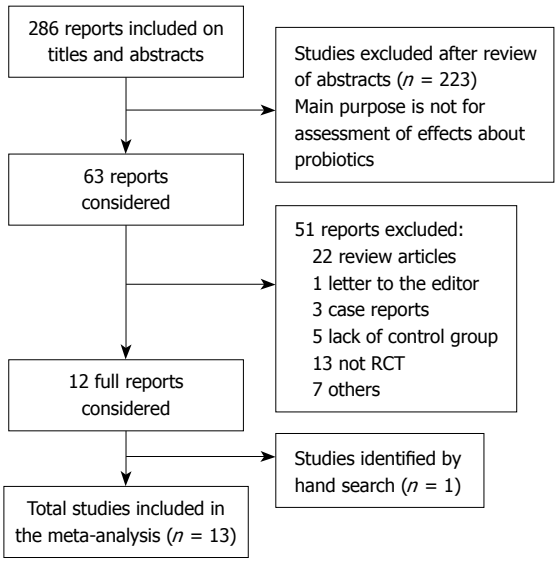

Figure 1 Study selection process. A flowchart was present in this figure and the flowchart summarizes the selection of studies including numbers and reasons of studies excluded.

the follow-up visits was 1 to 12 mo. All the papers were published in English. The general conditions of the selected research are shown in Table 1[8-20].

Quality assessment of selected papers

The quality assessment of the 13 selected papers is shown in Table 2. Three reports were level A, eight were level B, and two papers were level C.

Induction of remission of ulcerative colitis by probiotics

Seven reports evaluated the remission rate, which involved a total of 399 patients. Among the 399 patients, probiotics were used as an auxiliary therapy in 219 patients, and 180 patients were treated by standard therapy or placebo. Comparing the probiotics auxiliary therapy group with non-probiotics auxiliary therapy group, the remission rates were not significantly different (remission rate: 1.35, 95% CI: 0.98-1.85, $P = 0.07$), but there was an obvious heterogeneity in the results ($P = 0.02$, $I^2 = 62\%$). The total remission rate of the probiotics auxiliary therapy group was 68.2%, and for the non-probiotics auxiliary therapy group it was 60.4% (Figure 2A).

Sub-group analysis

Due to the heterogeneity of the total remission rate, subgroups were analyzed based on the severity of disease, placebo or not, different kinds of probiotics, and the span of probiotics therapy. The selected reports were first separated into a mild to middle subgroup and an active

Table 2 Methodological quality of the 13 RCTs

Study	Random method	Allocation concealment	Blind method	Lost or exit	Quality assessment	Inclusion/exclusion criteria	Outcome measurement
Miele *et al*[13], 2009	Right	Right	Double blind	Yes, use ITT analysis	A	Both	CAI by Lichtiger; EI score; HI score
Sood *et al*[12], 2009	Right	Right	Double blind	Yes, use ITT analysis	A	Both	DAI by Sutherland
Henker *et al*[15], 2008	Right	Non-described	Non-described	No	B	Inclusion criteria	CAI by Rachmilewitz
Zocco *et al*[20], 2006	Right	Non-described	Non-described	No	B	Both	CAI by Rachmilewitz; EI by Baron; HI by Truelove-Richard
Matthes *et al*[11], 2006	Right	Not used	Double blind	Yes, use ITT analysis	C	Not mentioned	DAI by Sutherland
Furrie *et al*[9], 2005	Right	Non-described	Double blind	No	B	Inclusion criteria	CAI by Walmsley; SI by Baron
Tursi *et al*[8], 2004	Right	Non-described	Non-described	Yes, use ITT analysis	B	Both	CAI by Lennard; EI score; HI score
Kruis *et al*[18], 2004	Right	Right	Double blind	Yes, use ITT analysis	A	Both	Scores according to Rachmilewitz
Kato *et al*[10], 2004	Right	Non-described	Non-described	No	B	Both	CAI by Lichtiger; EI by Harig, Scheppach; HI by Matts
Cui *et al*[16], 2004	Right	Not used	Non-described	No	C	Not mentioned	Not mentioned
Ishikawa *et al*[17], 2003	Right	Non-described	Non-described	No	B	Inclusion criteria	Exacerbation of clinical symptoms
Rembacken *et al*[14], 1999	Right	Non-described	Double blind	Yes, use ITT analysis	B	Inclusion criteria	Scores according to Rachmilewitz
Kruis *et al*[19], 1997	Right	Non-described	Double blind	Yes, use ITT analysis	B	Both	The same of the CAI score under the *E. coli* and mesalazine; Scores according to Rachmilewitz

CAI: Clinical activity index; SI: Sigmoidoscopy index; EI: Endoscopy; HI: Histology index.

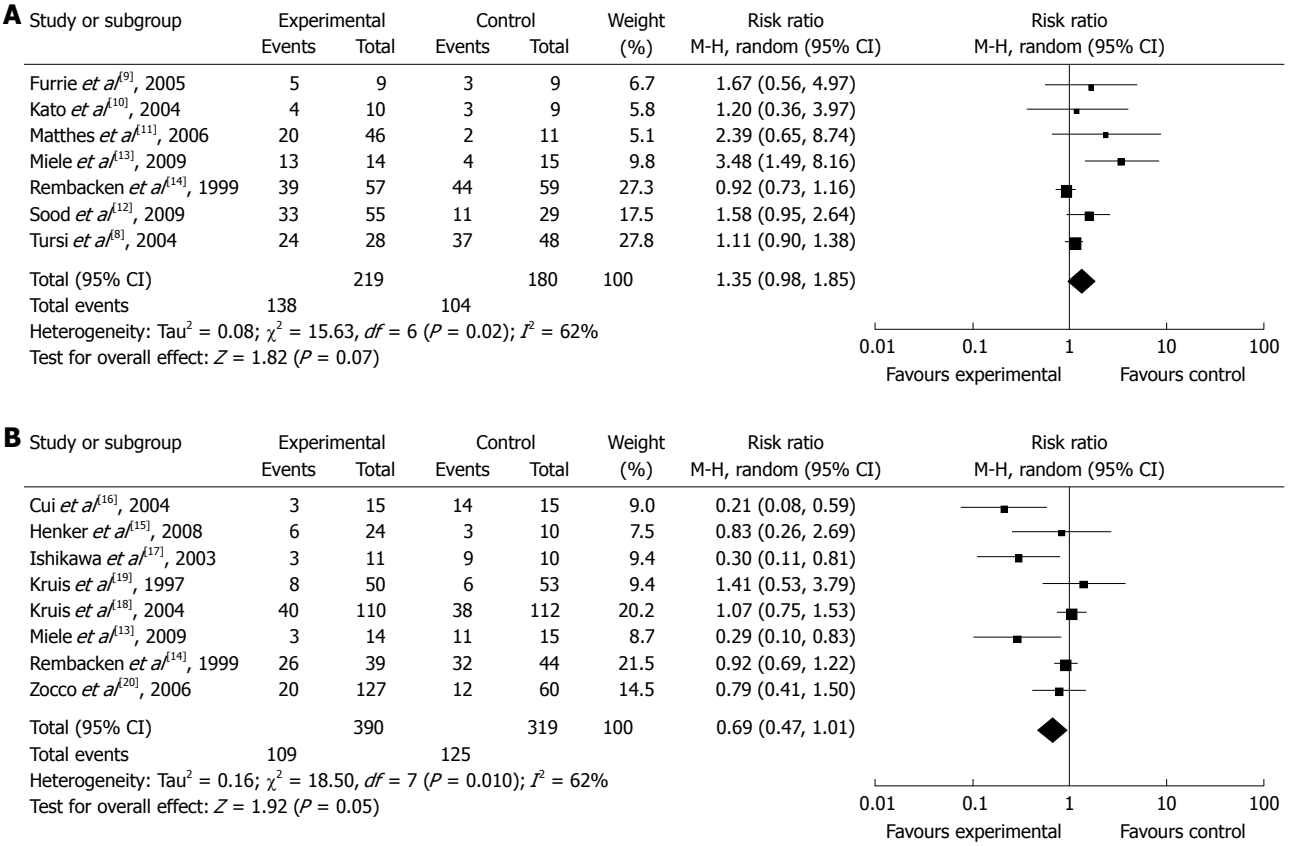

Figure 2 Remission rate (A) and relapse rate (B) of probiotic group *vs* control group.

phase subgroup. The remission rate of the mild to middle sub-group was 1.64 (95% CI: 0.98-2.72) and heterogeneity was obvious (P = 0.02, I^2 = 66%). The remission rate for the active phase subgroup was 0.97 (95% CI: 0.77-1.22), and heterogeneity existed (P = 0.28, I^2 = 13%). Secondly, the selected reports were divided into a placebo controlled sub-group and a non-placebo controlled sub-group. The remission rates of these two sub-groups were 2.00 (95% CI: 1.35-2.96) and 1.00 (95% CI: 0.85-1.18), respectively. The remission rate of the probiotics auxiliary therapy sub-group was significantly better than the placebo sub-group, and there were obvious heterogeneities in these two sub-groups (P = 0.46, I^2 = 0%). The selected reports were then separated into a VSL#3 subgroup, an *E. coli* subgroup, and a *Bifidobacterium longum* subgroup. The remission rate of the VSL#3 subgroup was 1.66 (95% CI: 0.87-3.15), and heterogeneity was obvious (P = 0.006, I^2 = 80%). The remission rate of the *E. coli* subgroup was 1.02 (95% CI: 0.80-1.30), and heterogeneity was obvious (P = 0.12, I^2 = 59%). The remission rate of the *Bifidobacterium longum* subgroup was 1.43 (95% CI: 0.64-3.19), and heterogeneity was not apparent (P = 0.69, I^2 = 0%). Finally, the selected research was divided into groups that received treatment for at least 12 mo or less than 12 mo, based on the course of treatment. The remission rate for the 12 mo sub-group was 1.69 (95% CI: 0.43-6.67), and heterogeneity was not apparent (P = 0.002, I^2 = 90%). The remission rate of the sub-group that received treatment for less than 12 mo was 1.36 (95% CI: 1.07-1.73). In the subgroup that received treatment for less than 12 mo and was treated by probiotics auxiliary therapy, the remission rate was obviously better than the control group, and heterogeneity existed (P = 0.33, I^2 = 14%).

Sensitivity analysis

Seven reports evaluated the remission rate. In one of the studies, the patients were treated by probiotic enema formulations but not by humatin, so this research was excluded. Sensitivity analysis indicated that the total remission rate was 1.30 (95% CI: 0.94-1.78), and that the statistical heterogeneity was significant (P = 0.02, I^2 = 63%). There was no significant differences in the remission rates between the groups that received probiotics or non-probiotics.

Effect of probiotics on the recurrence rate in maintenance treatment of ulcerative colitis

Eight reports assessed the recurrence rate, which involved a total of 709 patients, 390 of them used probiotics for maintenance treatment, and 319 patients used control medicine or a placebo. There was a significant difference between the total recurrence rates between the probiotics maintenance therapy group and placebo maintenance therapy group (recurrence rate: 0.69, 95% CI: 0.47-1.01, P = 0.05). The total recurrence rate for the probiotics maintenance therapy group was 27.9%, while the recurrence of the control group was 39.2%. The heterogeneity of the total recurrence rate was found to be significant by meta-analysis (P = 0.01, I^2 = 62%).

Subgroup analysis

Due to the significant heterogeneity of the total recurrence rate, it was necessary to analyze the subgroups to explain and/or reduce the heterogeneity. The selected studies were first divided into an active phase subgroup, a remission stage subgroup, and a mild to middle subgroup. The remission rate of the active phase subgroup was 0.48 (95% CI: 0.10-2.26) with obvious heterogeneity (P = 0.003, I^2 = 89%). The remission rate of the remission stage subgroup was 1.01 (95% CI: 0.14-0.61) without heterogeneity (P = 0.96, I^2 = 0%). The recurrence rate of the probiotics therapy group was much lower than the non-probiotics therapy group. The selected research was then separated into a placebo control subgroup and a non-placebo control subgroup. The remission rate of the placebo control sub-group was 0.25 (95% CI: 0.12-0.51) without obvious heterogeneity (P = 0.68, I^2 = 0%). The non-placebo control subgroup's remission rate was 0.92 (95% CI: 0.75-1.14) with visible heterogeneity (P = 0.26, I^2 = 24%), which was obviously higher than the placebo control sub-group. The remission rate of the probiotics therapy group was not significantly different from the non-placebo control sub-group.

The selected research was then grouped into an *E. coli* subgroup, a *Bifidobacterium* subgroup, and a VSL#3 *Lactobacillus* subgroup. The remission rate of the *E. coli* subgroup was 1.02 (95% CI: 0.81-1.29), and there was no obvious heterogeneity (P = 0.76, I^2 = 0%). The remission rate of the *Bifidobacterium* subgroup was 0.25 (95% CI: 0.12-0.51) without visible heterogeneity (P = 0.63, I^2 = 0%). The VSL#3 *lactobacillus* subgroup remission rate was 0.59 (95% CI: 0.35-1.01) with significant heterogeneity (P = 0.11, I^2 = 60%). Finally, the selected studies were divided into patients that had received treatment for 12 mo and those that had received treatment for less than 12 mo. The remission rate of the 12 mo subgroup was 0.83 (95% CI: 0.68-1.03) with significant heterogeneity (P = 0.07, I^2 = 51%), while the remission rate of the group that had received treatment for less than 12 mo was 0.55 (95% CI: 0.09-3.51) with significant heterogeneity (P = 0.009, I^2 = 85%) (Figure 2B).

Publication bias assessment

The publication bias could not only be qualitatively analyzed using a funnel plot to observe whether a bias existed, but we could also quantitatively count the bias level using a formula to determine the extent of the publication bias.

Quantification of publication bias: In the above analysis concerning probiotic treatment and its effect on the remission rate and recurrence rate of ulcerative colitis, the publication bias was analyzed quantitatively; however, quantification of the publication bias requires positive results, thus the total recurrence rate of ulcerative colitis affected by probiotics only was assessed.

To compare the recurrence rate between the probiotics group and non- probiotics group, the values used were as follows: ln (R) = -0.3711, W = 101.59, m = 8 × $(0.3711/1.96)^2$ × (101.59/8) - 8 = 21. The result showed

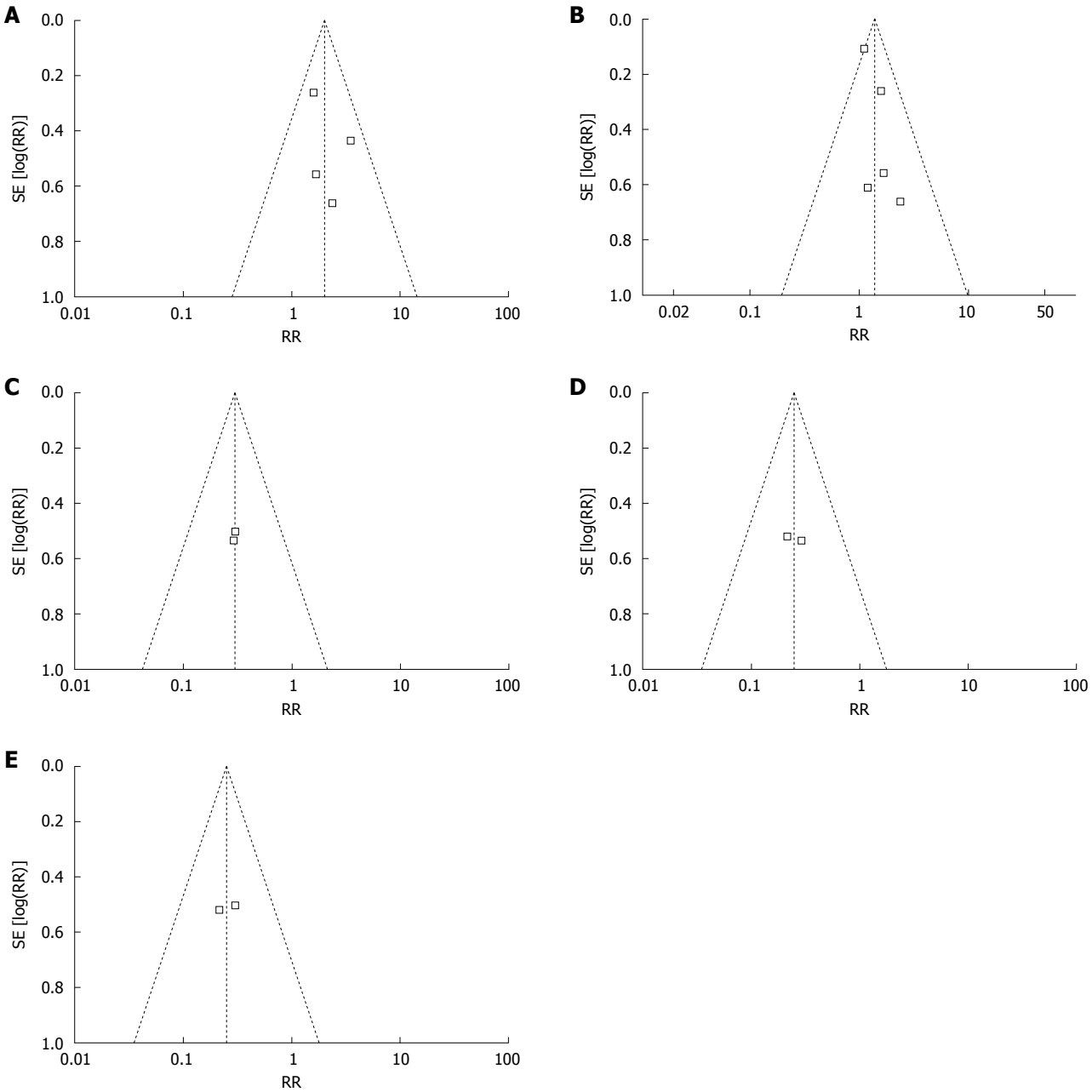

Figure 3 Inverted funnel plot analysis of the relative of remission rate. A: Inverted funnel plot analysis of the relative of remission rate between probiotics group and placebo group; B: Less than 12 mo group: Inverted funnel plot analysis of the relative of remission rate between probiotics group and non-probiotics group; C: Mild and middle UC group: Inverted funnel plot analysis of the relative of remission rate between probiotics group and non-probiotics group; D: Inverted funnel plot analysis of the relative of remission rate between probiotics group and placebo group; E: Inverted funnel plot analysis of the relative of remission rate between *Bifidobacterium longum* therapy group and non-probiotics therapy group.

the analysis results could not be reversed unless 21 non-statistically significant papers were added. This indicated that publication bias had little effect on the results in the analysis of recurrence rate of probiotics group and non-probiotics group. Therefore, the results were reliable.

Qualitative evaluation of publication bias: Inverted funnel plot analysis can analyze the results from a fixed effect model. Figure 3 shows that the inverted funnel plot had an uneven distribution and apparent asymmetry. This meant that in the patients who were treated for less than 12 mo, there was a publication bias in the relative of remission rate between the probiotics group and the non-probiotics group. The inverted funnel plots that were symmetrical indicated that there was no visible publication bias.

DISCUSSION

This study shows that using probiotics provides no additional benefit in inducing remission of ulcerative colitis, but probiotics auxiliary therapy is much better than non-probiotics therapy for maintenance therapy. Research has discussed the various effects of probiotics as an ulcerative colitis therapy. The mechanism of probiotics in ulcerative

colitis therapy mainly involves the following: (1) to prevent pathogen infiltration by restraining bacterial adherence and bacteria translocation, or to produce anti-bacterial substances that inhibit pathogenic bacteria; (2) to improve the intestinal mucosal barrier function, and the permeability and stability of the mucosal barrier; and (3) to regulate the mucosal immune response. Probiotics cause modifications of the mucosal immune response, improve activities of macrophages and NK cells, stimulate the production of antibodies, regulate the nuclear factor-κB (NF-κB) pathway, induce the apoptosis of T cells, and reduce the secretion of proinflammatory factors[21]. However, most of the studies on the effects of probiotics in ulcerative colitis therapy were performed *in vitro* or in animal models. Although probiotic effects are closely related to applied clinical conditions, there are some disputes between the results from basic research and clinical research. In addition, the inconsistency of research baselines has also caused disagreement among clinical researchers.

Although this study shows that using probiotics provides no additional benefit in the induction of remission for ulcerative colitis, there was a visible heterogeneity in the total remission rate. This effect might be related to the different methods used by the selected papers, such as the kinds of probiotics used, the treatment time, the types of ulcerative colitis, medication compliance of patients, whether patients were treated by antibiotics simultaneously, difference of the control groups, the analysis of the results, and the sample size. Some research shows that there are significantly different effects yielded by different kinds of bacteria and yeasts used as therapies[22,23]. Subgroup analysis demonstrated that the probiotics group was more likely to be in remission than the placebo group of patients with ulcerative colitis. In the subgroup that received treatment for less than 12 mo, the remission rate of the probiotics group was obviously higher than the non-probiotics group. The results suggested that probiotics auxiliary therapy could be used as another choice to induce the remission of ulcerative colitis, which has a better remission effect than the non-probiotics therapy group after 1 year. Some research has shown that the protective effects of probiotics act during the initial stage of ulcerative colitis impairment, but not during the refractory period. This result still needs to be confirmed using additional random control tests.

In the maintenance therapy stage, the probiotics auxiliary therapy was obviously better than non-probiotic therapy; that is, the recurrence rate of probiotics auxiliary therapy group was significantly lower than non-probiotics therapy group during the maintenance therapy stage, and this difference was heterogeneous. Subgroup analysis showed that the effect of probiotic maintenance therapy was obviously better than non-probiotic therapy in the mild to middle subgroup, placebo subgroup, and *Bifidobacterium* subgroup, and this difference was statistically significant. The results demonstrated that probiotics auxiliary therapy was more suitable in ulcerative colitis patients. Compared with standard treatments, such as 5-ASA or mesalazine, the effect of probiotics auxiliary therapy was not significantly different, but was obviously better than placebo therapy. The recurrence rate of the *Bifidobacterium* group was much lower than the non-probiotics therapy group, and the difference was statistically significant.

The main advantage of probiotics therapy is that it does not affect the regeneration of normal mucosa during active therapy[23]. To overcome gastrointestinal tract infections, probiotics must be non-pathogenic and resistant to antibiotics. In addition, probiotics can resist stomach acid, bile, and antibiotics and can modify immune processes to destroy invading microorganisms[24].

In this meta-analysis, the evaluation criteria of the results were not totally consistent. To a certain extent, this condition added to the heterogeneity of the systemic review.

In conclusion, whether the use of probiotics is better than non-probiotics therapy during the remission induction stage and maintenance stage of ulcerative colitis still needs further clinical trials, including variations of bacterial species, applied dosage, treatment timing, and the course of treatment.

COMMENTS

Background

Ulcerative colitis is a chronic recurrent colon disease whose pathogenesis is not clear. Whether probiotics should be used in treatment remains in dispute. This paper compares the effects of probiotics and drug treatment of ulcerative colitis or placebo in remission induction and maintenance of ulcerative colitis.

Research frontiers

Many studies indicated probiotics have positive effects in treating gastrointestinal diseases, including ulcerative colitis. However, the data comes from small sample studies and does not provide enough evidence of probiotics' advantage.

Innovations and breakthroughs

Thirteen randomized controlled experiments with relatively larger sample sizes, systematically evaluated the probiotics' remission induction and maintenance effects in treating ulcerative colitis.

Applications

Through meta-analysis, probiotics should be considered as an auxiliary medicine in the remission induction stage and maintenance stage of ulcerative colitis.

Peer review

This is a meta-analysis of the effect of probiotics on ulcerative colitis, which might provide some useful information. It is a nice, well-conducted study.

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BRIEF ARTICLE

Natural taurine promotes apoptosis of human hepatic stellate cells in proteomics analysis

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Abstract

AIM: To study the differential expression of proteins between natural taurine treated hepatic stellate cells and controls, and investigate the underlying regulatory mechanism of natural taurine in inhibiting hepatic fibrosis.

METHODS: A proteomic strategy combining two-dimensional gel electrophoresis and ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) was used to study the differential expression of proteins and Western blotting was used to validate the results. Gene ontology (GO) method was utilized to analyze the functional enrichment of differentially expressed proteins. Flow cytometry was performed to compare the apoptosis rate between taurine-treated and untreated hepatic stellate cells (HSCs).

RESULTS: Nineteen differentially expressed proteins (11 up-regulated and 8 down-regulated) were identified by 2D/MS, and the expression profiles of GLO1 and ANXA1 were validated by Western blotting. GO analysis found that these differentially expressed proteins were enriched within biological processes such as "cellular apoptosis", "oxidation reaction" and "metabolic process" in clusters. Flow cytometric analysis showed that taurine-treated HSCs had a significantly increased apoptosis rate when compared with the control group.

CONCLUSION: Natural taurine can promote HSC apoptosis so as to inhibit hepatic fibrosis.

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Key words: Natural taurine; Proteomics; Hepatic stellate cell; Hepatic fibrosis; Apoptosis

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INTRODUCTION

Hepatic fibrosis (HF) is the excessive accumulation of extracellular matrix (ECM) through which the liver repairs injury[1,2]. HF occurs in most types of chronic liver diseases due to various causes, and approximately 25%-40% of HF cases may ultimately progress to hepatic cirrhosis or even hepatocellular carcinoma. Therefore, prevention of the initiation of HF and interference with or reversal

of the fibrotic process is important for controlling the development of chronic liver diseases, which has become one of the research focuses in China and other countries[3,4].

Hepatic stellate cells (HSCs) are nonparenchymal cells found in the perisinusoidal space of the liver also known as the space of Disse[5]. HSCs contribute significantly to the occurrence of HF because activated HSCs have been found to secrete a great amount of ECM when migrating, proliferating and synthesizing in the injured site of the liver[6,7]. It is generally believed that reversal of the fibrotic process is difficult once HSCs are activated. Some studies, however, have shown that HF may be inhibited to a certain extent by reducing the number of HSCs *via* apoptosis induction[8,9].

Taurine, or 2-aminoethanesulfonic acid ($C_2H_7NO_3S$), is an important anti-injury substance in the body[10]. Taurine has a protective effect on various types of liver injury[11,12] and may mitigate HF by inhibiting the deposition of ECM[13]. The antifibrotic mechanism of taurine may involve its inhibition of the activation and proliferation of HSCs[9,14]. Because the molecular mechanism of taurine-mediated antifibrotic activity has not been fully unveiled and is little studied, it is imperative to use "omics" methods to systematically investigate the molecular mechanism by which taurine inhibits HF.

Therefore, in the present study, we analyzed the differential expression of proteins between taurine-treated HSCs and controls using two-dimensional electrophoresis (2-DE), ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) and bioinformatics method to identify proteins associated with the inhibition of HF by taurine. Moreover, we aimed to understand better the molecular mechanism underlying taurine's protection against HF and thereby provide new targets for the management of HF and drug development.

MATERIALS AND METHODS

Materials

Human HSCs (LX-2) were purchased from XiangYa Central Experiment Laboratory, Central South University, Changsha, Hunan Province, China. Natural taurine was provided by Yuanlong Pearl Co. Ltd., Beihai, Guangxi Zhuang Autonomous Region, China. Acrylamide, bisacrylamide, glycine and HRP-ECL luminescent substrate were purchased from Sigma (USA). Immobilized pH gradient (IPG) strips (immobiline™ DryStrip, pH3-10, nonline, 18 cm) and image analysis software (ImageMaster 2D platinum) were purchased from Amersham Pharmacia. Bradford protein assay kit was provided by Beyotime Institute of Biotechnology (China). Primary and secondary antibodies were purchased from Santacruz; Annexin V apoptosis assay kit, from Nan Jing KeyGen Biotech Co., Ltd. (China); vertical electrophoresis apparatus and Trans-Blot system, from Bio-Rad (USA); Epics XL Ⅱ flow cytometer, from Beckman Coulter (USA); inverted light microscope, from Olympus (Japan); IPGphor IEF System and ImageScanner, from Amersham Pharmacia. Vertical slab electrophoresis apparatus (PROTEAN Ⅱ xi Cell) was bought from Bio-Rad (USA) and UPLC-ESI-MS/MS system was provided by Micromass (UK).

Methods

Incubation and treatment of HSCs (LX-2): The cells were incubated in DMEM containing 100 U/mL penicillin, 100 U/mL streptomycin and 10% fetal bovine serum at 37℃, 50 mL/L CO_2 and saturated humidity. The medium was replaced every other day. When the cell density achieved 80%-90%, trypsin was used for digestion and the cell concentration was adjusted to be 1×10^5/mL. The supernatant was discarded and then reaction was maintained for 48 h after the addition of 2% DMEM containing 40 mmol/L taurine. HSCs were randomized equally to receive treatment with or without taurine.

Protein extraction and quantification: The cells were collected after centrifugation and washed in PBS twice. After 10 μL of RNAase and 10 μL of DNAase, were added respectively, the solution was placed in ice bath at 4℃ for 20 min and then centrifuged at 14000 × *g* for 30 min and the supernatant was collected. Protein quantification was performed by the Bradford method. In the same way, 1 μL of the sample was collected and 99 μL of water was added followed by supplementation with 1 mL of Bradford working solution. After shaking for 5 min, measurement was conducted. The absorbance of the test sample was measured at 595 nm.

2-DE: The first-dimension isoelectric focusing (IEF) electrophoresis was performed on an IPGphor IEF System. Proteins were centrifuged for 2 min before loading. A loading amount of 150 μg was dissolved in rehydration solution containing 8 mol/L urea, 0.02% Chaps, 0.02 mol/L dithiothreitol (DTT) and 0.05% IPG buffer and then 800 μL of cover fluid was added. The focused gel strips were equilibrated twice in sodium dodecyl sulfate (SDS) equilibration buffer (1.5 mol/L tris-Cl, pH 8.8, 50 mmol/L, 30% Glycerol, 6 mol/L Urea, 2% SDS, Bromophenol blue trace) and rotated on a shaking table for 15 min × 2. The first equilibration buffer was supplemented with 20 mmol/L DTT and the second equilibration buffer was added with 100 mmol/L iodoacetamide. Gel strips were taken out and then the vertical second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run on a PROTEIN Ⅱ xi Cell system. Polyacrylamide gels (13%) were used for separation. Electrophoresis was run at a constant current of 40 mA for 40 min and at 60 mA for 5 h until the bromophenol blue front reached the bottom of the gel. Rapid silver staining was performed in accordance with the manufacturer's manual. Gels were stained with 0.1% Coomassie brilliant blue R350 at 100℃ for 10 min and then de-stained in 10% acetic acid overnight.

Gel image analysis: Stained 2-DE gels were scanned in transmission scan mode using an Image-scanner™. Then,

matching of protein spots and differential expression analyses between the treatment group and the control group were performed using ImageMaster 2D Platinum 5.0 software.

Mass spectrometry and database query: In-gel digestion was done for 19 differentially expressed protein spots whose concentrations met the requirement of mass spectrometry (MS). In-gel digested products were analyzed by UPLC-ESI-MS/MS (Micromass Company). The mass spectral data obtained after processing with the data analysis software PLGS v2.3 were used to search through the NCBInr database with a MS/MS ion strategy using Mascot software (http://www.matrixscience.co.uk). The search parameters included trypsin digestion, M oxidation and iodoacetamide alkylation (variable modifications), and 1 missed cleavage. The mass error tolerance in both the MS and MSMS data was 0.2-Da.

GO functional analysis: GO is a stratified tree structure for analysis of gene and protein functions[15]. We used the BinGO plugin in the Cytoscape platform to analyze functions of 19 differentially expressed proteins, with special focus on the enrichment of these proteins in biological process[16,17]. Then, we classified the significantly enriched GO terms according to functional similarity. Finally, we visualized the significantly enriched GO terms and their parent/child items in a tree structure in the Cytoscape platform and indicated the classification of the GO terms in the diagram.

Apoptosis detection: HSCs were incubated in the presence of 40 mmol/L taurine for 48 h, washed in PBS twice and then re-suspended in 100 μL of buffer containing calcium ion. After this, staining was carried out for 20-30 min by adding 5 μL of Annexin V-FITC dye and then for 5 min by supplementation with 5 μL of PI dye. The cell concentration was adjusted to be about 1×10^5/mL by adding a proper amount of calcium ion-containing buffer. The cells were detected on a flow cytometer within 1 h, with the excitation wavelength and the emission wavelength of 488 nm and 530 nm, respectively. The experiment was repeated in triplicate for each sample.

Western blotting: Total proteins in HSCs were extracted as described in the section of protein extraction and quantification, quantified by the Bradford method, isolated by SDS-PAGE and then transferred onto a nitrocellulose membrane with a Trans-Blot SD apparatus. The membrane was blocked in a blocking buffer (TBST solution containing 5% skim milk) at room temperature (RT) for 2 h. Primary antibodies were added at a dilution of 1:500 and the membrane facing down was incubated at RT for 1 h with slight shaking. After incubation with primary antibodies, the membrane was washed and incubated at RT for 1 h with mild shaking in the presence of horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibodies (1:5000). After this, the membrane was washed. *Visualization* of *protein bands* was achieved by the chemiluminescence method and the films were developed and fixed. GAPDH was used as an internal reference. The experiment was repeated in quadruple for each protein.

Statistical analysis

Data were expressed as mean ± SD. Difference in the relative content of differentially expressed protein spots was analyzed by *t* test for statistical significance ($P < 0.05$) and *t* test was also performed to determine whether treatment with taurine caused a statistically significant change in apoptosis of HSCs ($P < 0.05$). Hypergeometric test was used to test the enrichment of GO terms. A multiple testing procedure controlling false discovery rate (FDR) was performed as described by Benjamini and Hochberg, with the threshold for enrichment significance set to be FDR < 0.01[18]. All statistics were performed on R software.

RESULTS

2-DE and image analysis

Under the same experimental conditions, the 2-DE experiment was repeated 3 times separately for 2 groups of protein samples. Three pieces of parallel gels with high resolution and good reproducibility were obtained for each group. The mean number of protein spots from 3 pieces of gels was 745 ± 32 in the taurine treatment group *vs* 748 ± 25 in the control group, with a match rate of 86.08% between groups. Six hundred and forty-three differential protein spots were screened from both groups. Of these protein spots, 19 spots (11 up-regulated and 8 down-regulated) showed more than three-fold differential expression (Figure 1) following treatment with taurine. Figure 2 is a local amplification of 4 pairs of up-regulated or down-regulated protein spots, which clearly demonstrates changes in protein expression between taurine-treated HSCs and controls.

Identification of differentially expressed proteins by UPLC-ESI-MS/MS

Identification by UPLC-ESI-MS/MS was performed for the 19 differentially expressed proteins following in-gel digestion and peptide extraction. The resulting peptide fragments were used to search through the NCBInr database using Mascot software and these 19 differentially expressed proteins were successfully identified (ions scores > 38, $P < 0.05$). Table 1 summarizes the code, name, relative molecular weight, isoelectric point and peptide fragment coverage for the differentially expressed proteins. The most significantly up-regulated expression was observed in GLO1, a glyoxylase capable of catalyzing and forming lactoylglutathione, which has been reported to be linked to inflammatory reactions and tumors[19,20]. In contrast, AKR1A1, an aldehyde reductase important for both glucose metabolism and oxidative reactions[21], showed the most markedly down-regulated expression.

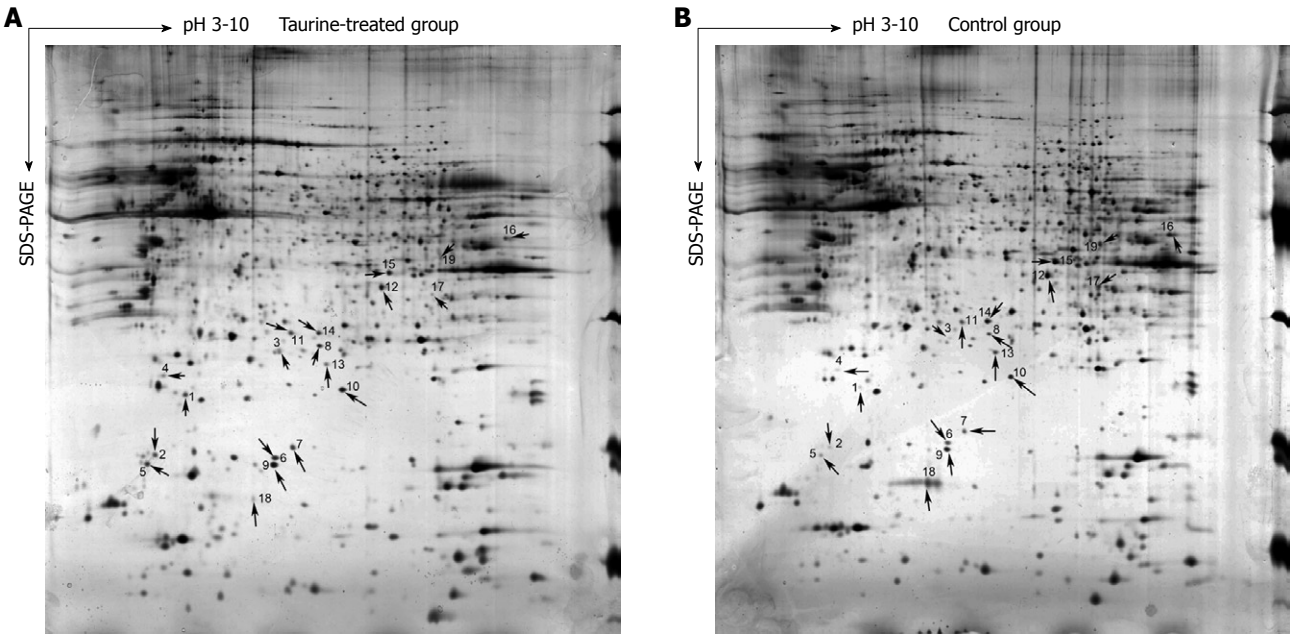

Figure 1 Two-dimensional gel electrophoresis (2-DE) images of taurine-treated hepatic stellate cells (HSCs) and controls. A: Taurine-treated group; B: Control group. In both images, transverse arrows represent isoelectric focusing and longitudinal ones represent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

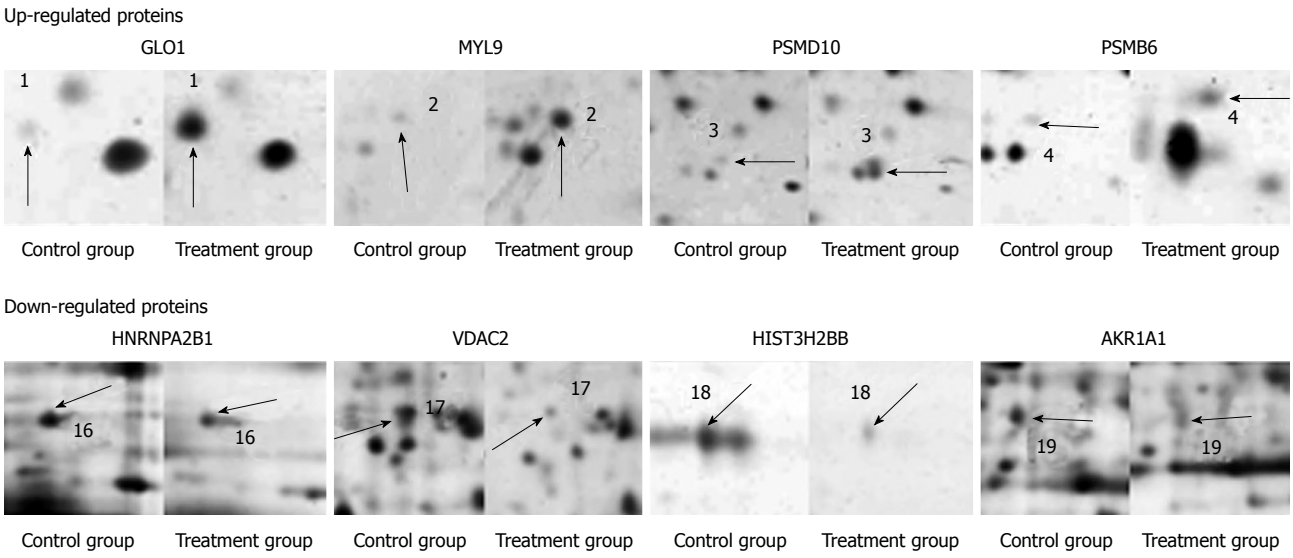

Figure 2 Local amplification of some differentially expressed protein spots (arrows). Up-regulated: GLO1, MYL9, PSMD10 and PSMB6, and down-regulated: HNRNPA2B1, VDAC2, HIST2H2BB and AKR1A1.

Validation of differentially expressed proteins by Western blotting

Among the differentially expressed proteins identified successfully by mass spectrometry, the protein GLO1 and ANXA1 were selected and subjected to Western blotting detection. Taurine treatment caused a significantly up-regulated expression of GLO1 and ANXA1 in HSCs (Figure 3). These expression profiles were completely consistent with the locally amplified 2-D electrophoretogram in Figure 2. Therefore, Western blotting detection of the differentially expressed proteins confirmed the reliability and validity of the proteomic high throughput experiments.

GO functional analysis of differentially expressed proteins

We entered the names of the differentially expressed proteins into the BinGO plugin in the Cytoscape platform to perform GO functional enrichment analysis, with special focus on the changes in the biological process of HSCs after taurine treatment. Table 2 lists 9 significantly enriched GO terms and relevant proteins identified after screening with a threshold of FDR < 0.01. Functional categorization of the GO terms revealed that these differentially expressed proteins were enriched within biological processes such as "cellular apoptosis", "oxidation reaction" and "metabolic process" in clusters. Figure 4 provides

Table 1 MS identification of differentially expressed proteins in taurine-treated and control groups

Code	Protein spot ID	Record number of protein	Name of protein	Description	log_2 (differential ratio)	Relative molecular weight (kDa)	Isoelectric point	Score	Coverage (%)
Up-regulated proteins									
1	1394	gi|2392338	GLO1	Chain a, human glyoxalase i with benzyl-glutathione inhibitor	4.25	20.6	5.12	408	51
2	1431	gi|29568111	MYL9	Myosin regulatory light chain 9 isoform a	3.30	19.8	4.80	631	62
3	1330	gi|4506217	PSMD10	Proteasome 26S non-ATPase subunit 10 isoform 1	3.24	24.4	5.71	279	35
4	1380	gi|558528	PSMB6	Proteasome subunit Y	2.62	25.3	4.80	403	26
5	1438	gi|4507669	TPT1	Tumor protein, translationally-controlled 1	2.48	19.6	4.84	375	61
6	1428	gi|4502101	ANXA1	Annexin I	1.48	38.7	6.57	1069	53
7	1419	gi|5174539	MDH1	Cytosolic malate dehydrogenase	1.18	36.4	6.91	223	17
8	1321	gi|4504517	HSPB1	Heat shock protein beta-1	0.99	22.8	5.98	567	49
9	1432	gi|5453710	LASP1	LIM and SH3 protein 1	0.95	29.7	6.61	362	34
10	1385	gi|31543380	PARK7	Parkinson disease protein 7	0.70	19.9	6.33	128	50
11	1285	gi|2982080	SOD1	Chain a, familial als mutant g37r cuznsod	0.50	15.9	5.87	292	45
Down-regulated proteins									
12	1130	gi|5453559	ATP5H	ATP synthase, H+ transporting, Mitochondrial F0 complex, subunit d isoform a	-0.66	18.5	5.21	277	47
13	1355	gi|5802974	PRDX3	Peroxiredoxin 3 isoform a precursor	-0.70	27.7	7.67	128	16
14	1284	gi|194097323	ECHS1	Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor	-0.83	31.4	8.34	481	30
15	1067	gi|9955007	PRDX2	Chain a, thioredoxin peroxidase b from red blood cells	-1.14	21.8	5.44	446	38
16	953	gi|14043072	HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform B1	-1.86	37.4	8.97	181	15
17	1165	gi|190200	VDAC2	Porin	-2.81	38.1	6.32	281	22
18	1464	gi|1568551	HIST3H2BB	Histone H2B	-3.19	13.9	10.31	270	35
19	980	gi|5174391	AKR1A1	Aldo-keto reductase family 1, member A1	-3.55	36.6	6.32	306	22

MS: Mass spectrometry.

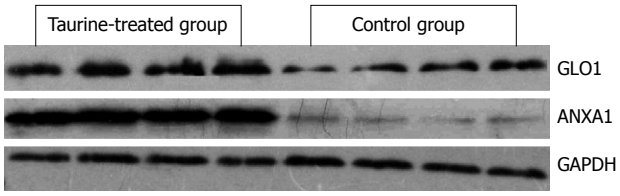

Figure 3 Results of validation by Western blotting for differentially expressed proteins. GAPDH was used as an internal reference. The experiment was repeated 4 times for each protein. Four columns in the right side represent the control group and the other 4 columns in the left side are from the taurine-treated group.

a visualized network of the 9 significantly enriched GO terms and their parent/children items. We can see that Cluster 1, 2 and 3 correspond to the category of "cellular apoptosis", "oxidation reaction" and "metabolic process" in Table 2, respectively. The consistency between these two dimensions suggests an important role of these biological processes in taurine-treated HSCs.

Results of apoptosis detection

To further confirm the functional findings from proteome data, we detected apoptosis of HSCs treated with or without taurine. Figure 5 shows that taurine treatment resulted in a significant increase in the apoptosis rate of HSCs (13.6% ± 3.3% *vs* 4.65% ± 1.1% in the control group, $P < 0.05$). Thus, proteome data and results of apoptosis detection consistently suggest that taurine may have an inhibitory effect on HF by promoting HSC apoptosis.

DISCUSSION

In the present study, we employed a proteomics strategy combining 2-DE and UPLC-ESI-MS/MS to investigate difference in protein expression between natural taurine-treated and untreated HSCs. Nineteen differentially expressed proteins (11 up-regulated and 8 down-regulated) were identified. The protein GLO1 and ANXA1 were then selected and subjected to validation by Western blotting, yielding expression profiles in full agreement with results obtained by 2-DE. GO functional analysis showed that these differentially expressed proteins were enriched within biological processes - "cellular apoptosis", "oxidation reaction" and "metabolic process" in clusters. Flow cytometric analysis showed that taurine-treated HSCs had a significantly increased apoptosis rate compared with the control group. Our comparative proteomic analysis revealed that taurine may inhibit HF by promoting HSC apoptosis.

The apoptosis mechanism of HSCs is complex where various apoptotic pathways are not only inter-independent but also interconnected. For instance, the iron chelator deferoxamine, which may increase the activity of caspase 3 when inducing the release of mitochondrial cytochrome C (cyt-c), induces HSC apoptosis *via* both the mitoch-

Table 2 Functional categorization of differentially expressed proteins

GO number	Functional description	Proportion (%)	Protein	*P* value	FDR value
Apoptosis-related					
51093	Negative regulation of developmental process	32	TPT1 | ANXA1 | HSPB1 | GLO1 | PRDX2 | SOD1	2.20E-6	2.61E-4
42981	Regulation of apoptosis	32	TPT1 | ANXA1 | HSPB1 | GLO1 | PRDX2 | SOD1	3.19E-5	1.49E-3
43067	Regulation of programmed cell death	32	TPT1 | ANXA1 | HSPB1 | GLO1 | PRDX2 | SOD1	3.45E-5	1.49E-3
Oxidation reaction-related					
42542	Response to hydrogen peroxide	16	PRDX3 | SOD1 | PARK7	4.28E-6	4.07E-4
6979	Response to oxidative stress	21	PRDX2 | PRDX3 | SOD1 | PARK7	8.18E-6	5.65E-4
302	Response to reactive oxygen species	16	PRDX3 | SOD1 | PARK7	8.33E-6	5.65E-4
Metabolism-related					
44248	Cellular catabolic process	37	PSMB6 | AKR1A1 | PSMD10 | ECHS1 | PRDX3 | SOD1 | MDH1	9.67E-6	5.74E-4
9056	Catabolic process	37	PSMB6 | AKR1A1 | PSMD10 | ECHS1 | PRDX3 | SOD1 | MDH1	3.12E-5	1.49E-3
42743	Hydrogen peroxide metabolic process	11	PRDX3 | SOD1	2.45E-4	9.69E-3

Hypergeometric test, FDR < 0.01. GO: Gene ontology.

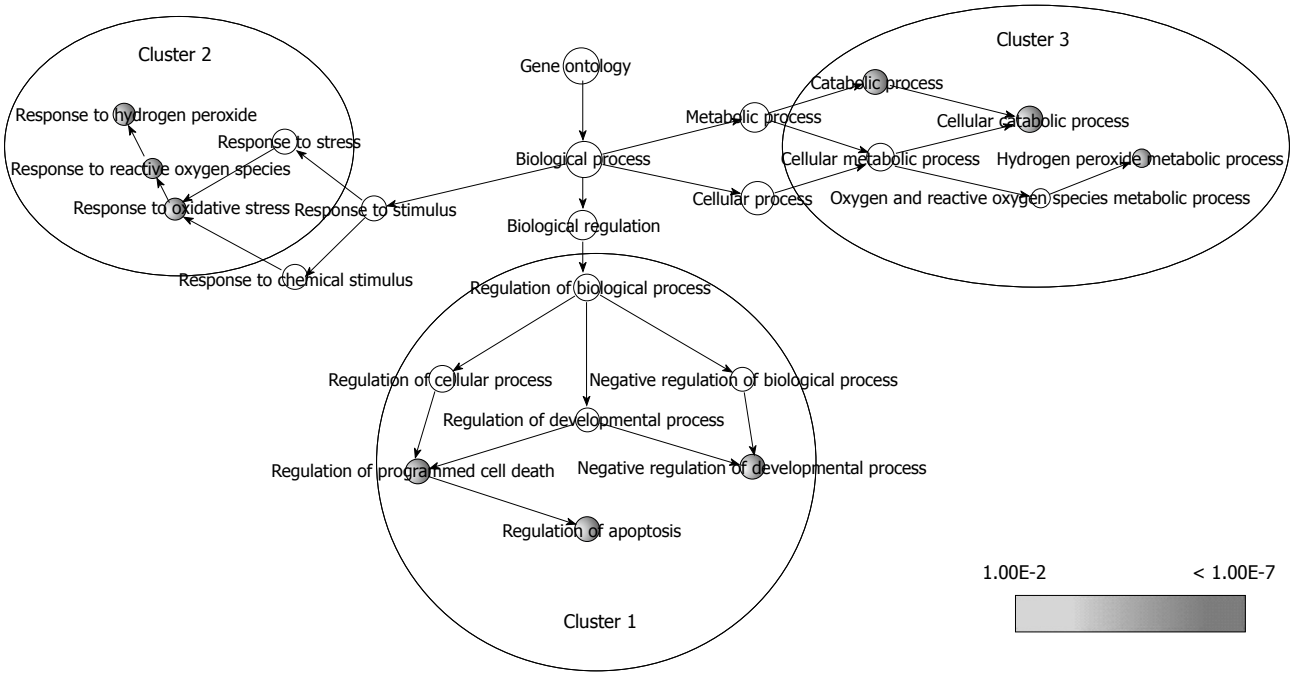

Figure 4 Network diagram of functional categorization of differentially expressed proteins. The categories in this tree structure fully correspond to the 3 categories in Table 2 and the depth of color ranging from light to dark gray indicates the significance level of FDR in Table 2. Gray nodes represent significantly enriched GO terms and white nodes signify GO terms that have a parent/child relationship with gray nodes. Connecting lines between nodes construct a directional hierarchy of the GO terms.

ondrial pathway and the death receptor pathway[22]. Our previous study has also found that natural taurine may regulate the TGF-β1/smad signaling pathway and has an activity against HF[23]. To date, however, the molecular mechanism and relevant pathways responsible for taurine's ability to promote HSC apoptosis have not been well understood. During confirmation of this phenomenon, we found differential expression of proteins including TPT1, ANXA1, HSPB1, GLO1, PRDX2 and SOD1 in taurine-treated HSCs, which was tightly associated with cellular apoptosis. Therefore, we presume that TPT1, ANXA1, HSPB1, GLO1, PRDX2 and SOD1 are important proteins in the taurine-mediated pathway of HSC apoptosis. These proteins may play a role in classical apoptotic pathways. Or they may constitute, on their own, a specific apoptotic pathway for HSCs by interacting with each other and regulating other proteins. We need to analyze taurine-mediated HSC apoptosis based on findings. Our another previous work has shown that natural taurine may protect mitochondria in HF rats by regulating liver lipid peroxidation, indicating an anti-HF effect[24]. Functional analysis of the differentially expressed protein demonstrated the ability of taurine to regulate oxidation reaction in HSCs. In fact, oxidation-related biological processes, such as reactive oxygen species (ROS) in Table 2, are closely associated with apoptosis induction[25]. Among

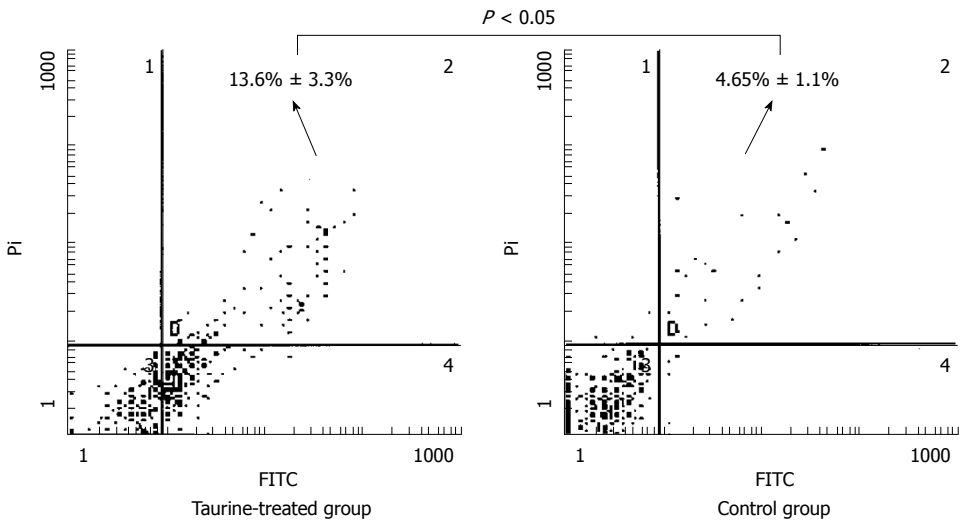

Figure 5 Apoptosis profiles of HSCs treated or untreated with taurine. The experiment was repeated 3 times for each sample (*t* test, $P < 0.05$). Cell count in the second quadrant was used to calculate the apoptosis rate.

the proteins involved in oxidation reaction, some may be engaged in HSC apoptosis as well (SOD1), and some belong to the same protein family (PRDX3 and PRDX2). Moreover, the hydrogen peroxide metabolic process listed in Table 2 is actually one of the basic steps of oxidation reaction[26,27] and this metabolism-related process also involves SOD1 and PRDX3. Thus, it is possible that, in the interconnected process of HSC apoptosis, SOD1 and PRDX3 play a critical mechanism-specific role. For the reasons given above, we presume that natural taurine may facilitate the release of cyt-c from mitochondria and then promote HSC apoptosis by directly or indirectly inducing activation of caspase *via* ROS[25,28] and that SOD1and PRDX3 are two significant functional proteins in the process.

The hepatic parenchyma consists of parenchymal liver cells (PLCs) including hepatocytes and non-parenchymal liver cells (NPLCs) associated with sinusoids including HSCs. It has been demonstrated that the activation of HSCs is the key issue in the pathogenesis of HF[7]. Changes of hepatocytes proliferation may stimulate hepatocyte interaction with HSCs possibly *via* cell adhesion molecules leading to HSC activation and HSC clustering[29,30]. It is obvious that HSCs is the most important HF target cell line in the liver, however, HSC-HSC and hepatocyte-HSC cell adhesion may play a combined role in HF. Therefore, study of the activation and apoptosis of HSC can extensively survey the possible pathological mechanisms of HF, but further investigations on animal models or clinical patients *in vivo* will obtain more exact results for HF in living organisms.

Taurine can either be synthesized chemically or extracted from natural sources. However, natural taurine is more superior to synthetic taurine in promoting HSC apoptosis[31]. The advantage and innovativeness of our present study are reflected in the selection of natural taurine for investigation of its mechanism in regulating HSC apoptosis. In addition, since no studies using proteomic high throughput technologies to investigate the inhibition of HF by taurine have been published in China and other countries, our findings are useful for future systematic studies on biological mechanism.

Life is a vast and complex dynamic network system and the therapeutic mechanism of drug treatment mimics an integrated modulatory chain effect involving a variety of pathways in regulating biological networks[32]. The present study, which systematically investigated how natural taurine may regulate HF-associated biological networks, will help to cast a new light on the action mechanism and targets of taurine.

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COMMENTS

Background

Hepatic fibrosis (HF) occurs in most types of chronic liver diseases and approximately 25%-40% of HF cases may ultimately progress to hepatic cirrhosis. Hepatic stellate cells (HSCs) contribute significantly to the occurrence of HF and the activation of HSC is the key issue in the pathogenesis of HF. It is expected that HSCs are targets for pharmacological or molecular interventions for the treatment of HF.

Research frontiers

Taurine is a kind of important anti-injury substance in the body. Taurine has a protective effect on various types of liver injury. It has been clear that the antifibrotic mechanism of taurine may involve its inhibition of the activation and proliferation of HSCs. However, the molecular mechanism of taurine-mediated antifibrotic activity is largely unknown. Now the research hotspot is to unveil the underlying regulatory mechanisms and accumulate data for drug development.

Innovations and breakthroughs

In the present study, the authors analyzed the differential expression of proteins between taurine-treated HSCs and controls by comparative proteomics technologies. It is the first investigation to study the natural taurine mediated protection effects against HF. Nineteen differentially expressed proteins were identified and functional analysis provided confident evidence of HSC apoptosis promotion by taurine treatment.

Applications

The study results are very helpful to understand better the molecular mechanism underlying taurine's protection against HF and thereby provide new targets for the management of HF and drug development.

Peer review

The authors investigated the effect of taurine on the HSCs by proteomic analysis. They identified 19 differently expressed proteins belonging to the

biological process of apoptosis, cellular oxidation and metabolic processes. They also found an increased rate of apoptosis in taurine exposed cells. Overall, this work is of interest in relation to taurine induced protection in HF and in the underlying alterations in specific proteins in HSC.

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CASE REPORT

Cystic fibrosis and Crohn's disease: Successful treatment and long term remission with infliximab

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Abstract

The association of cystic fibrosis and Crohn's disease (CD) is well known, but to date, there are very few cases in the literature of patients suffering from mucoviscidosis who have required treatment with infliximab. We report the case of a 23-year-old patient suffering from cystic fibrosis and severe CD treated successfully with infliximab without any infective complications or worsening of the pulmonary disease and with a long term (2 years) complete remission.

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Key words: Cystic fibrosis; Crohn's disease; Infliximab

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INTRODUCTION

The association of cystic fibrosis (CF) and Crohn's disease (CD) is well known, but, to date, there are very few cases in the literature of patients suffering from mucoviscidosis who have required treatment with infliximab.

CF is the most common life-threatening autosomal recessive disease in Caucasian children; it has an incidence of 1 case in every 2500 children born alive[1]. CF involves an anomalous function of the exocrine glands, caused by a mutation of a gene (cystic fibrosis transmembrane conductance regulator, *CFTR*) located on chromosome 7, which codes for a protein involved in ion transport through the cell membrane[1]. More than 1000 mutations of *CFTR* have been identified, the most common of which is ΔF 508, found in 50% of CF patients. Pulmonary complications are the most common causes of mortality, but the presenting symptoms are very often linked to gastrointestinal and pancreatic biliary diseases. These are mainly caused by the unusual viscosity of the secretions in hollow organs and in the ducts of solid organs[1].

Meconium ileus, intussusception, appendicitis, rectal prolapse, gastro-esophageal reflux, CD and fibrosing colonopathy are the gastrointestinal diseases observed in patients suffering from CF[1,2].

CD is a chronic inflammatory bowel disease which may be localized throughout the gastrointestinal tract. The association between CD and CF is known; there are reports of a prevalence of CD in patients suffering from CF 17 times higher than in controls[3].

CD in patients suffering from CF is, therefore, not an exceptional event; the use of an immunosuppressant such as infliximab in patients suffering from CF may, however, be considered as uncommon, as seen in the lack of data available in the literature. Probably, underlying this lack of

data is the fear that the immunosuppressive properties of such a biological treatment would be contraindicated by the characteristic infections in CF, especially of the lung.

We report the case of a 23-year-old patient suffering from CF and severe CD who was treated successfully with infliximab and who is in long term remission.

CASE REPORT

This is a report of the case of a 23-year-old female suffering from CF who, from the age of 16 years, started having recurrent abdominal pain associated with weight loss. She was regularly followed up by a centre for CF.

At the age of 14 years, she underwent explorative laparotomy with appendicectomy for suspected acute abdomen. The operation was complicated by the appearance of a cutaneous fistula at the site of the surgical wound. Because of persistence of symptoms associated with severe deterioration of nutritional condition, she was sent to our centre. A low digestive endoscopy carried out at our unit showed a condition of acute pancolitis compatible with chronic inflammatory bowel disease. The histological examination of the multiple biopsies taken confirmed the suspected diagnosis of CD.

An induction treatment cycle with prednisone, full dose for 4 wk, with mesalazine and metronidazole was initiated. Having obtained clinical and endoscopic histological remission, we reduced the prednisone dose, stopped metronidazole and continued maintenance treatment with mesalazine. The patient continued treatment with mesalazine alone with good clinical progress for about four years during which she did not come to us for check-ups but preferred to go to the hospital in her city of residence.

In 2003, she underwent emergency laparotomy at another centre following the appearance of acute abdomen. Surgery showed a pericolic abscess collection in the cecum and ascending colon; she was then subjected to ileocecal resection and ileotransverse colonic anastomosis. At two months following the surgery, the girl was treated with steroids, azathioprine and mesalazine for severe flare-up of disease. She continued with low dose steroid treatment (5-10 mg/d) for about two years; treatment with azathioprine was suspended owing to poor efficacy and was replaced with methotrexate (MTX).

During this period of time the patient presented stable respiratory function (spirometry, clinical outcome of exacerbation), but she developed chronic *Pseudomonas aeruginosa* airway infection. Therefore, ciprofloxacin or trimethoprim sulfamethoxazole or doxycycline was administered for 2 wk twice every 3 mo.

In August 2006, the patient returned to us spontaneously following recurrent episodes of abdominal pain and considerable weight loss (about 10 kg in the previous 2 mo). An ileocolonoscopy carried out under sedoanalgesia showed hyperacute disease of the sigmoid-rectum (Figure 1A and B). Multiple "tags" were present in the anal site with a big "knife-cut" lesion at the level of the posterior commissure. The histological examination confirmed a severe reactivation of her disease. The

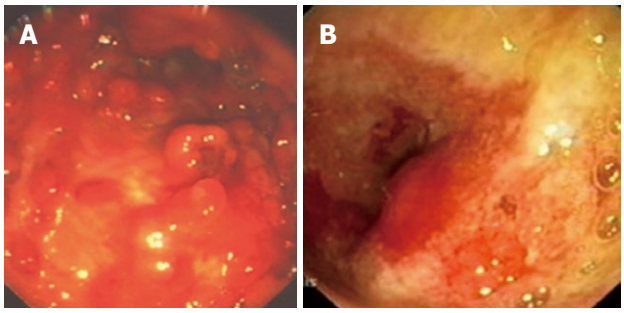

Figure 1 Ileocolonoscopy findings. A: Multiple inflammatory pseudopolyps; B: Hyperacute Crohn's disease of the sigmoid-rectum.

CD activity index (CDAI) was 390. Index values of 150 and below are associated with quiescent or non-active disease (i.e. "remission"); values over 150 are indicative of active disease, and over 450, extremely severe disease[4]. Total parenteral nutrition was therefore started with complete suspension of fractionated feeding by oral administration and medical treatment was resumed (cortisone, metronidazole as antibiotic and mesalazine as anti-inflammatory).

Due to the steroid therapy, fasting blood glucose pathologically increased (320 mg/dL) and therefore subcutaneous insulin therapy was started with an improvement of glucose intolerance.

Endoscopic histological examination performed after a short time (4 wk), showed only modest improvement of the lesions found earlier during the endoscopic examination, despite improvement of the general condition.

Because of the severity of the lesions, particularly of the anus, and after informing the patient and the pneumologists, we decided to start a biological treatment cycle with infliximab in addition to treatment with azathioprine (AZA) (2.5 mg/kg per day) in a single administration in the morning, associated with antibiotic treatment with 3rd generation cephalosporin and glycopeptide. The infusions of infliximab can be superimposed with this treatment regime, which is used in our centre, with an initial treatment at 0, 2, and 6 wk, followed by a maintenance phase with infusions every 8 wk.

At the discontinuation of steroids, insulin therapy was no longer necessary.

After the first three intravenous infusions of infliximab, endoscopic examination showed complete regression of anal lesions and distinct improvement of the lesions of the sigmoid rectum, with healing appearance of the mucosa and no continuous lesions up to the ileocolic anastomosis.

The appearance of the ileum, explored to about 40 cm, was within acceptable limits. Nine months after beginning the treatment and after 7 infusions of infliximab, the patient showed distinct improvement in her general condition and body weight increased by 13 kg.

Twelve months after commencement of the treatment, condition continues to be optimal and there has been no complication involving infection in the lung or septic episodes. The working of the lungs was kept stable during

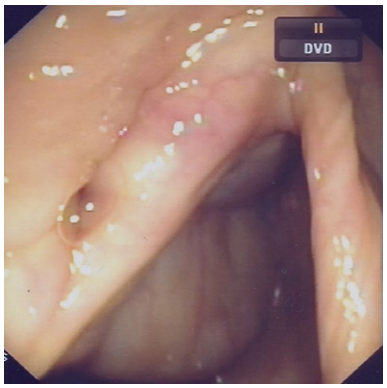

Figure 2 Endoscopic remission.

the course of treatment with infliximab (forced expiratory volume in 1 s (FEV1) 85% in April 2006 and FEV1 80% in July 2007), without any pulmonary exacerbations.

Suspension of the biological treatment was therefore programmed after the 10th infusion, while treatment with AZA and mesalazine as maintenance continued. Since the last infliximab infusion two years have passed and a complete remission of CD has been maintained both clinically and endoscopically (Figure 2).

DISCUSSION

This report deals with the case of a patient suffering from CF who, at the age of 16 years, was diagnosed as suffering from CD. The literature reports frequent cases of CD during the course of CF. The prevalence is about 17 times greater than that of controls[3]; this shows that there is a pathogenic relationship between the two diseases. The etiopathogenesis has not yet been identified but a number of mechanisms have been proposed, the most probable of which is an altered immune response to a chronic infection[4]. Furthermore, patients who are carriers of the ΔF 508 mutation seem to have an increased risk of developing gastrointestinal problems[5].

The literature contains case reports of diagnosis of CD in patients suffering from CF who complain of long periods of abdominal pain and most of all, loss of weight, in spite of appropriate nutritional and therapeutic support[6,7]. As in the case of our patient, protracted gastrointestinal symptoms and lack of response to basic treatment make it necessary to conduct instrumental examinations, in particular endoscopic examinations such as gastroscopy and colonoscopy, associated with perendoscopic biopsies, which allow a differential diagnosis between CD and fibrosing colonopathy[7,8]. This has led, in the last few years, to an increase in the diagnosis of CD during the course of CF[7], which was not suspected in the past. The aim of treatment during the course of CD is to induce and maintain remission of the disease. Corticosteroids have demonstrated a high efficiency in inducing clinical remission in patients with moderate to severe forms of disease[9,10]. In order to maintain the remission and to reduce the risk of dependence on corticosteroids, there is general agreement regarding the early introduction of immunosuppressants such as AZA, MTX, 6 mercaptopurine (MP) and also infliximab[11-13]. In the case of our patient, given the modest clinical and endoscopic response after one month of treatment with steroids, this therapeutic opportunity was also discussed with the pneumologists. Biological treatment with infliximab associated with AZA was therefore started, because of the presence of significant anal lesions, although the literature currently contains few case reports of patients suffering from CF treated with infliximab. However, numerous studies have shown the efficacy of infliximab in inducing or maintaining remission of disease in adults suffering from CD who have moderate-severe forms, with perianal localization or with fistulizing forms[9,10,14-17]. In patients with CF the presence of bronchiectasis and colonization with *Pseudomonas*, as in our patient, would normally represent a relative contraindication to the administration of infliximab, but numerous studies of bronchoalveolar lavage fluid from patients with CF have shown a high concentration of inflammatory mediators such as tumor necrosis factor α (TNFα)[18]. Indeed, drugs such as azithromycin ameliorate lung function in cystic fibrosis patients while also reducing the levels of TNFα[19].

In our experience with this patient, we observed a significant clinical improvement even after the first dose. This improvement was endorsed by the resolving of endoscopic and histological lesions, as is also reported in the literature[9,20,21]. The association of infliximab with AZA was justified by the fact that concurrent treatment with AZA, 6 MP or MTX may be helpful in maintaining the clinical response to infliximab, reducing the amount of circulating antibodies directed towards the latter; which are thought to be responsible for some cases of non response[22-25].

As shown in the literature[18,19], as well as in the case of our patient, the moment a treatment focused for CD was introduced, although not free of risks, a distinct improvement of the general condition was observed with weight increase of about 13 kg. After over 12 mo of treatment with infliximab and AZA, no pulmonary complication which would have compelled suspension of treatment was observed. In addition, the literature shows that no severe adverse events have occurred and that there was no reported increase in the prevalence of respiratory tract infections during infliximab administration in patients with chronic obstructive pulmonary disease[26].

The main questions arising from treatment with infliximab in this patient were, firstly, the possibility that the biological treatment could increase susceptibility to infections by opportunistic pathogens given the concurrent basic lung disease, since patients with cystic fibrosis already spontaneously suffer from such infections. Moreover, the infections most frequently reported during infliximab infusions are respiratory tract infections and urinary tract infections (35% infliximab-treated patients *vs* 25% placebo recipients)[27].

Secondly, given the excellent clinical, endoscopic, and histological progress of the patient, there was doubt regarding the best moment for suspension of the drug,

especially taking into consideration the possibility of causing allergies or reduced efficacy in case of re-treatment because of the possible presence of antibodies against infliximab[9,22,23,25,28,29].

As regards the first challenge, the constant cover with antibiotic treatment was found to be effective in keeping the lung disease under control, as it did not worsen but rather remained stable as is shown by the spirometry tests taken during the course of immunosuppressive treatment.

As regards suspension of treatment, in the absence of precise bibliographic guidance, we believed that it was appropriate to suspend treatment in accordance with previously reported data after a period of not less than one year[30].

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CASE REPORT

Use of biological meshes for abdominal wall reconstruction in highly contaminated fields

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Abstract

Abdominal wall defects and incisional hernias represent a challenging problem. In particular, when a synthetic mesh is applied to contaminated wounds, its removal is required in 50%-90% of cases. Biosynthetic meshes are the newest tool available to surgeons and they could have a role in ventral hernia repair in a potentially contaminated field. We describe the use of a sheet of bovine pericardium graft in the reconstruction of abdominal wall defect in two patients. Bovine pericardium graft was placed in the retrorectus space and secured to the anterior abdominal wall using polypropylene sutures in a tension-free manner. We experienced no evidence of recurrence at 4 and 5 years follow-up.

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Key words: Biological meshes; Bovine; Pericardium; Infection; Hernia

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INTRODUCTION

Abdominal wall defects and incisional hernias represent a challenging problem. The risk of developing an incisional hernia after a midline laparotomy is up to 11%[1]. The size of the abdominal wall defect and the potential presence of contamination of the site can complicate this commonly performed surgical repair. Several techniques have been adopted over time. Primary repairs often lead to unacceptable high tension, and their recurrence rate has been reported as high as 12%-50%[1,2]. In patients whose fascial defect is significant, mesh repair is preferable in order to obtain a tension-free abdominal wall closure. In the latter case, the recurrence rates are reported between 3% and 24%[2-4]. According to the literature, the use of prosthetic mesh reduces the recurrence rate but is also associated with serious complications in 10%-15% of cases. Some of these complications, such as infection, fistula and skin erosion, often lead to mesh removal[4]. In particular, when

a synthetic mesh is applied to contaminated wounds, its removal is required in 50%-90% of the cases[5]. Strength, flexibility, host tissue compatibility and ability to avoid infections should characterize an ideal mesh. Many synthetic and biological mesh tissues have been proposed over time but no single material, nor newer biosynthetic mesh, has fulfilled these requirements and gained universal acceptance. We present two cases in which biological meshes were used successfully in contaminated fields.

CASE REPORT

Case 1

A 62-year-old white woman presented to the General Surgery and Senology Unit of our University with a 6-mo history of abdominal pain after right hemicolectomy for carcinoma through a midline laparotomy at another institution. Her medical history was also significant for obesity, hypertension and coronary artery disease. She stated that pain occurred soon after the surgical procedure, together with random fever. Six months later, she developed an abdominal wall abscess that spontaneously drained externally through a small incisional hernia in the right-upper abdomen, and formed a persistently draining sinus. Following this, she underwent incisional hernia repair with pre-peritoneal prolene mesh placement. After 4 mo, she developed a small fistula again (Figure 1A), therefore, she was admitted to our division for further care. On physical examination, we observed a well-healed midline incision with mild tenderness along the incision itself. Computed tomography (CT) of the abdomen showed a multiloculated collection around the mesh, which measured approximately 12 cm × 15 cm. A course of antibiotic therapy (imipenem) was initiated before bacterial cultures were obtained. The first culture was positive for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The second, after 1 wk of appropriate antibiotic administration, remained negative for any microorganism.

We scheduled the patient for exploration and mesh removal. We entered the abdominal cavity through her previous midline laparotomy. The cavity was then exposed and the fluid collection aspirated along with debris. The inflammatory process extended to the periostium of the tenth rib. Removal of the mesh from the anterior abdominal wall required extensive and tedious dissection. The abdomen was entered and all adhesions to abdominal organs (bowel and omentum) were removed. We noticed an anomaly of the rib profile, therefore, after further investigation, we discovered a bone fracture with surrounding tissue rearrangement. We excised the rib for histological examination and it was consistent with osteomyelitis (Figure 1B and C). After removal of the contracted mesh, we created skin flaps and re-positioned the posterior rectus sheath. We placed a sheet of bovine pericardium graft (Tutomesh®; Tutogen Medical, Germany) in the retrorectus space and secured the mesh in a tension-free manner to the abdominal wall, using polypropylene sutures. The anterior rectus sheath was then re-positioned with Vicryl, and the skin was closed with staples (Figure 1D). The patient had no significant postoperative complications, and she was discharged on postoperative day 6. We performed CT at 5 mo after surgery, which showed no seroma and excellent remodeling of the host tissue. There was no clinical evidence of recurrence at her 5-year follow-up visit.

Case 2

A 69-year-old man with a previous history of left hemicolectomy for diverticular disease underwent elective incisional hernia repair with intraperitoneal polytetrafluoroethylene (PTFE) mesh placement 8 mo later. Postoperatively, the patient developed a persistent infected seroma, which was unresponsive to antibiotic treatment. The patient underwent surgery again to drain the collection, debride the area, and substitute the contracted Goretex mesh with a polypropylene mesh in the retrorectus space. This attempt proved unsuccessful and led to abscess formation after only 1 mo. The patient was scheduled for elective removal of the polypropylene mesh. After removal of the contracted mesh (Figure 2A-C), the posterior rectus sheath was re-positioned to the midline, and a sheet of bovine pericardium mesh (Tutomesh®) was implanted in the retrorectus space and secured to the anterior abdominal wall using polypropylene sutures in a tension-free manner. The anterior abdominal wall was then re-positioned with Vycril interrupted sutures and the skin was closed with staples (Figure 2D). The patient had no significant postoperative complications, and he was discharged on postoperative day 3. At his 4-year follow-up visit, there was no evidence of recurrence.

DISCUSSION

Treatment of incisional hernias

Incisional hernias are still a challenging problem for the surgeon. Their primary repair leads to high recurrence rates (up to 50%) because of the tension created and myocutaneous flap necrosis[1,2].

Many techniques have been proposed over time to reduce tension, such as relaxing incisions, and compartment release. Unfortunately, the results are far from being optimal[6,7]. Furthermore, large, full-thickness, abdominal wall defects secondary to wide resection of malignant tumors, traumatic injuries, or congenital abnormalities, cannot be closed primarily. The use of prosthetic meshes has then become necessary. Along with the traditional open techniques of mesh implantation, the more recent laparoscopic approach has gained popularity because of the decrease in wound infection, recurrence rates and recovery time.

Unfortunately the intrinsic property of tissue ingrowth of the prosthetic meshes currently used, such as polypropylene, polyester and PTFE, is also the cause of unwanted adhesions, chronic sinus (2%-6%), fistula formation (0%-2%) and wound infections (2%-17%)[8]. Other potential complications of synthetic meshes in-

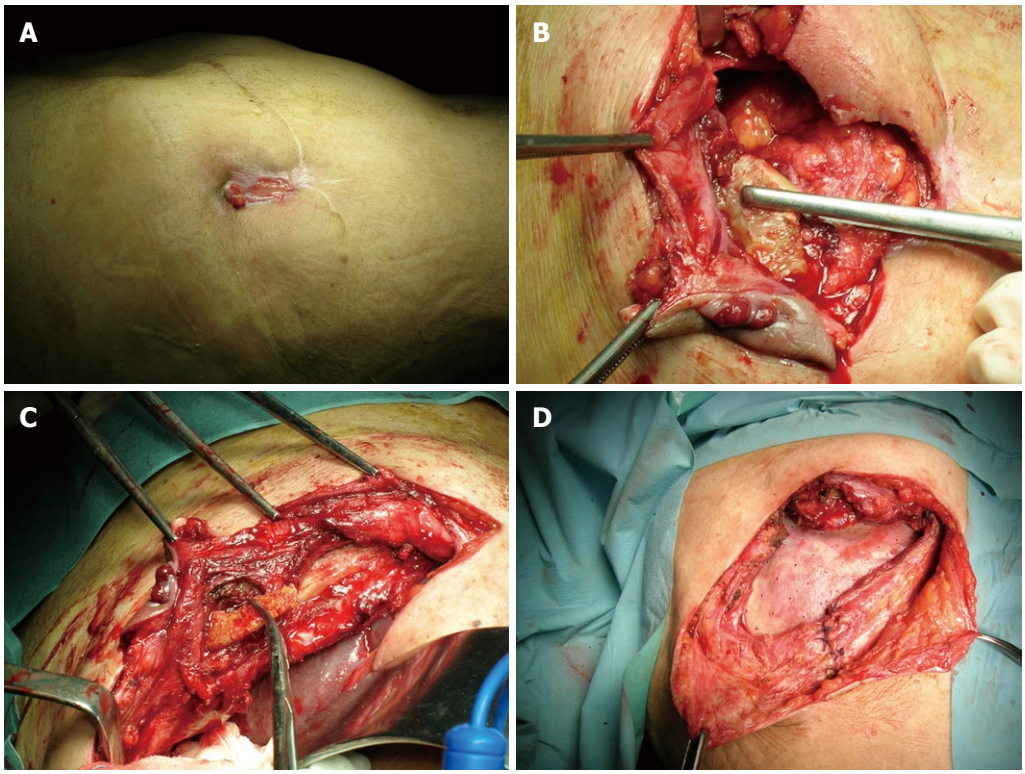

Figure 1 Case 1. A: Abdominal wall with subcutaneous fistula; B: Rib anomaly; C: Bone fracture with tissue rearrangement; D: Sheet of bovine pericardium mesh; anterior rectus sheath re-positioned with Vicryl.

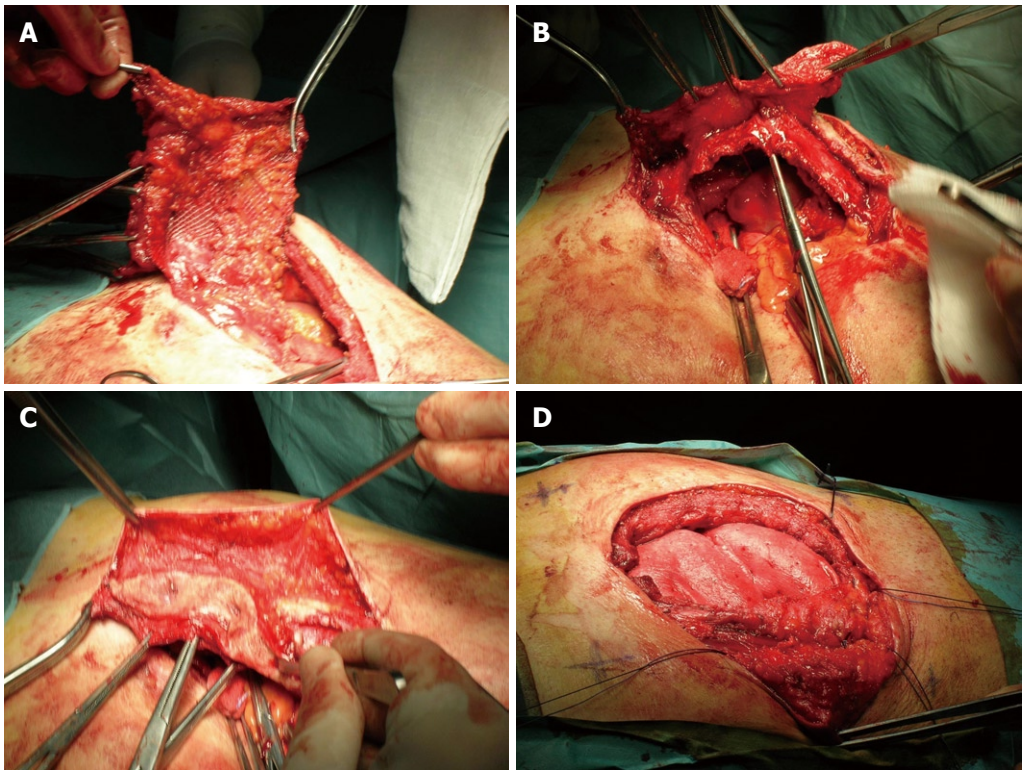

Figure 2 Case 2. A-C: Preexisting contracted mesh; D: Sheet of bovine pericardium mesh in the retrorectus space.

clude mesh contraction, migration, folding of the edges with visceral contact, recurrence, inflammation, seroma, and chronic pain due to inflammatory response and/or nerve entrapment. As a result, complications are common following ventral hernia repair, and occur in 5%-30% of laparoscopic cases and 27%-34% after open cases[9].

Table 1 Classification of biological grafts

Species of origin	Allogenic	Xenogenic	
Type of collagen matrix	Dermis	Intestinal submucosa	Bovine pericardium
Decellularization process	Yes	Yes	Yes (TUTOMESH®)
Cross-linking	Yes	No	No
Sterilization method	Salt solution, patented non-denaturing agents		H_2O_2, NAOH γ irradiation
Storage (need for refrigeration)	Yes	No	No
Rehydration requirement	Yes	No	No

Contaminated abdominal wall defect: role of biological prostheses

Even more challenging is the repair of complex contaminated abdominal wall defects, since no ideal operative technique or material for fascial reconstruction is currently available. The fear of fibrosis, erosions, infection and fistulas with the commonly used prosthetic meshes, has led engineers and surgeons to investigate alternative materials in order to achieve tension-free repair in a single-stage operation, even in highly contaminated fields. Biosynthetic grafts seem to offer a solution to this challenging problem. Their concept is to provide a collagen and other extracellular matrix scaffold, in which the host fibroblasts can create angiogenesis and deposit new collagen. The non-synthetic nature of these products allows them to be more resistant to infections. Several biological grafts are available on the market. Their classification is based on the species of origin (allogenic, xenogenic), type of collagen matrix utilized (dermis, pericardium, intestinal submucosa), decellularization process, presence or absence of cross-linking, storage requirements (need for refrigeration or not), and the need for rehydration (Table 1).

Bovine pericardium (TUTOMESH®) is a cadaveric bovine pericardium surgical mesh that is processed by solvent dehydration followed by gamma irradiation. It has no risk for transmission of viruses or prions. The graft consists of collagenous connective tissue with three-dimensional intertwined fibers, it has multidirectional mechanical strength, and can be implanted regardless of the direction of the graft. Collagenous connective tissue with multidirectional fibers retains the mechanical strength and elasticity of the native tissue, while providing the basic structure to support replacement by new endogenous tissue (known as remodeling). It is indicated for use in general, gynecological, cardiac and plastic surgery, such as repair of pericardial structures, soft tissue deficiencies, rectal and vaginal prolapse, and hernias (including diaphragmatic, femoral, incisional, inguinal, lumbar, paracolostomy, and umbilical hernias), gastric banding, muscle flap reinforcement, and reconstruction of the pelvic floor.

A bioprosthesis derived from human acellular dermal matrix (Alloderm®; Lifecell Corporation, Brachburg, NJ, USA) has been used as a tissue substitute for skin grafting over the past 20 years, but its use for abdominal wall reconstruction is relatively recent. At present, there are not enough data or long-term reports to establish universal consensus for its use over other bioprostheses, but it is the material with the most published literature. Some authors have described concerns about the laxity of the material and its ability to stretch over time, with the development of eventration or pseudo-recurrences[9].

Another available product is derived from porcine dermal collagen (Permacol™; Tissue Science Laboratories plc, Covington, GA, USA). It is a flexible acellular cross-linked porcine dermal collagen that is approved by the US Food and Drug Administration for use as a tissue substitute. It has been used in the United Kingdom since 1998. In addition to its three-dimensional collagen architecture that is similar to human dermis, it supports fibroblast infiltration and neovascularization. As a result of its manufacturing peculiarity of cross-linking, its remodeling process is delayed in the host tissue, which provides additional strength, but also possible incomplete remodeling. The porcine dermal collagen graft is easy to handle and has demonstrated comparable tensile strength to polypropylene. Integration into host tissue and neovascularization allow for antibiotic diffusion and in turn, greater resistance to infections, as in other similar biological grafts[10].

Surgical techniques: overview

Four types of incisional hernia repair with mesh have been commonly described.

In the inlay technique, the fascial defect cannot be approximated, so the prosthetic material is secured to the edges of the fascia to achieve abdominal wall closure. The onlay technique places the mesh above a primary fascial closure. The more recently described sublay technique (Rives Stoppa repair) applies the concepts of a tension-free technique in which the prosthetic mesh is secured in the retrorectus space after closure of the posterior rectus sheath/peritoneum. The aim is to prevent contact between the mesh and abdominal viscera. The coverage of the mesh provided by the muscle decreases the complications related to the mesh proximity to the subcutaneous tissue (seroma, wound infection)[11,12]. Finally, with the advent of meshes designed with an anti-adhesive barrier on one side, the mesh position has been moved one layer deeper, into the peritoneal cavity; the so-called intraperitoneal technique. In the latter, the mesh is secured to the abdominal wall with stitches, staples or tacks, and this is commonly performed laparoscopically.

Review of the literature

The data available on biological mesh implants for ventral hernia repair are very recent, and mostly in the form of case series. Lindermann *et al*[13] have published their experience using bovine pericardium graft in 37 incisional and two parastomal hernias. They have described four recurrences in the incisional group treated with the inlay technique (fascial defect cannot be approximated). They have suggested that the high elasticity of the mesh during the

remodeling phase could be the likely cause of recurrence. Post *et al*[14], in their series of 13 successful incisional hernias with sublay bovine pericardium, have described one case in which the mesh had to be re-attached after it had pulled off the suture line during a coughing spell. No infection, pain, foreign body feeling or recurrence was seen. Parker *et al*[10] have reported their experience using Permacol in nine patients affected by complicated abdominal defects with either a contaminated wound or a history of a hernia mesh infection. In all cases, the mesh was placed in the retromuscular underlay position, and in spite of the presence of active infection or gross contamination, no infectious complications occurred. Baghai *et al*[15] have evaluated 20 biological mesh implants in 17 patients who required recurrent ventral hernia repair following synthetic mesh explantation due to infection. Different biological grafts were utilized in the study (including porcine intestinal submucosa, human and porcine dermis). Also, all the implants were carried out with an open technique in the inlay, onlay or retrorectus position. All the patients treated with the inlay or onlay technique (n = 17) experienced wound infection and/or dehiscence within 2 wk of surgery. The three remaining biological implants, placed in the retrorectus space with closure of the anterior fascia, were successful with no infection, dehiscence or reccurrences at 6 mo follow-up. Therefore, Baghai *et al*[15] have suggested that biological mesh implants are not effective when used with an inlay or onlay technique. According these preliminary results, the mesh position, regardless of the type of biological mesh composition and texture, may play a key role in the incidence of hernia recurrence. Patients whose defect is corrected by a bridging technique (inlay technique) seem to have an increased tendency to develop eventration and a higher recurrence rate, compared with those whose defect is reinforced after fascia closure (sublay technique)[9]. According to some authors, the use of biological meshes in a bridging manner (inlay technique) provides only a temporary means for restoration of abdominal wall integrity in highly contaminated fields, which leads to future implantation of a more durable prosthesis to reestablish the integrity of the abdominal wall. Conversely, when the biological mesh is used to reinforce a primary fascia closure (sublay or underlay technique), the rate of recurrence seems comparable with other prosthesis[16,17]. In the setting of a contaminated wound, delayed closure has been proposed to prevent a closed environment infection and limit recurrence, but its routine use has not gained wide support[18].

Choice of biological prosthesis

The scarcity of literature comparing the different types of biological grafts precludes an evidenced-based decision about which one to use. Therefore, we have to decide according to the characteristics of the grafts to choose the appropriate product.

Permacol is available in larger sizes than cadaveric graft (up to 18 cm × 28 cm), it is less expensive than human cadaveric dermal graft, and does not require rehydration before being placed[10].

Bovine pericardium meshes have been used safely even for the repair of inguinal or paraesophageal hernias, in which the constant movement of structures is a risk factor for recurrence[19]. In our opinion, one of the main advantages of bovine pericardium graft resides in the relative lack of elastin compared to dermal products (both allogenic and xenogenic). This, in turn, results in a higher ratio of mature collagen/elastin at the end of the remodeling, possibly minimizing eventration and pseudo-recurrence[9]. Also, these products are easy to handle, do not require refrigeration, have a long shelf life, and can be used immediately after opening without long rehydration processes. Finally, they tend to be less expensive than their human tissue counterparts. However, further studies with larger numbers of patients and longer follow-up are required to guide better the right choice of material[9,20].

In conclusion, complex reconstruction of the abdominal wall can be challenging. Biosynthetic meshes are the newest tool available in the surgeon's armamentarium and they could have a role in ventral hernia repair in clean and contaminated fields. Their use is based on the concept of providing a collagen and extracellular matrix scaffold in which the host fibroblasts can create angiogenesis and deposit new collagen. In reviewing our results, the bovine pericardium as a xenogenic implant is a useful alternative to allogenic materials in incisional hernia repair. Further experience and longer follow-up is necessary to determine the results of bioprosthesis when used for abdominal wall reconstruction.

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CASE REPORT

Gastric adenomyoma presenting as melena: A case report and literature review

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Abstract

Gastric adenomyoma (AM) is a rare benign tumor characterized by gland-like structures embedded within a smooth muscle stroma. We report a case of a 68-year-old man with gastric AM admitted to our hospital for melena. Endoscopic examination revealed a gastric mass of about 4 cm in diameter, located in the antrum. Histologic examination of the excised specimen showed irregularly arranged glands and interlacing smooth muscle bundles surrounding the glandular elements. Although gastric AM is rare, it should be considered in differential diagnosis of extramucosal gastric tumor.

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Key words: Adenomyoma; Stomach; Melana; Histopathology; Endoscopic examination

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INTRODUCTION

Gastric adenomyoma (AM) is a rare, benign tumor, characteristically composed of glands and cysts, lined by columnar, flattened epithelia and a prominent smooth muscle stroma. The entity was first described by Magnus-Alsleben[1] in 1903. Patients with gastric AM may be asymptomatic, or have nonspecific gastrointestinal symptoms such as epigastric pain, vomiting[2-5]. We describe, herein, an extremely rare case of gastric AM presenting as melana.

CASE REPORT

A 68-year-old man was referred to our hospital with a 1-week history of melena. He had no remarkable past medical history and alcohol consumption or history of smoking or drugs. Upon hospitalization, physical examination, routine laboratory parameters and tumor markers including carcinoembryonic antigen, cancer antigen 19-9, and alpha fetoprotein were normal, except for markers of hypochromic anemia including hemoglobin 89 g/L (normal range: 120-160 g/L), hematocrit 28.6% (normal range: 40%-50%), mean corpuscular hemoglobin concentration 311 g/L (normal range: 320-360 g/L), serum ferritin 8.31 μg/L (normal range: 21.81-274.66 μg/L).

Abdominal computed tomography (CT) scanning showed a heterogeneous mass in the gastric antrum. Tumor tissue was slightly enhanced after injection of a

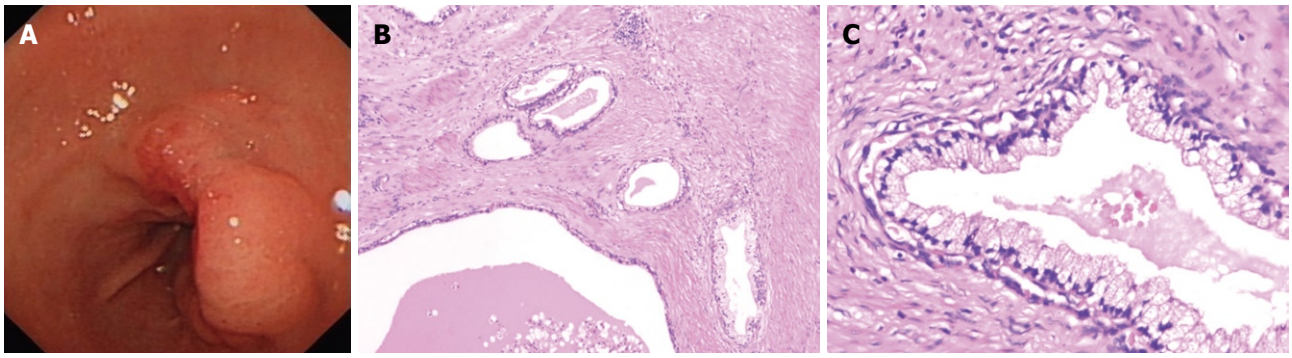

Figure 1 Gastric adenomyoma. A: Upper gastrointestinal (GI) endoscopy showing an irregular lesion with central ulcer in the gastric antrum; B: Microscopy showing several glands and cyst surrounded by smooth muscle (HE, × 50); C: High-power view showing glands lined by columnar micinous epithelium (HE, × 200).

contrast medium. Neither further invasion beyond the gastric wall, nor visible metastatic lesions in the liver or enlarged lymph nodes were observed.

Endoscopic examination revealed an ulcerated mass of about 4 cm in diameter, located on the lesser curvature and posterior wall of the gastric antrum, with the duodenum partly involved (Figure 1A). The esophagus and the remaining parts of stomach were normal. Histopathological examination of mass biopsies showed several dilated glands with mild atypia in an inflamed stroma and necrosis. A decision was made to proceed with surgery in order to relieve the symptoms. As frozen sections revealed the possibility of a gastric AM, a distal subtotal gastrectomy was performed. Postoperative recovery was uneventful.

Macroscopic examination revealed a 50 mm × 43 mm × 23 mm ulcerated mass with sharp margins and a greyish-white cut surface. It was solid and cystic with clear, mucinous fluid in the lumens. The cysts ranged up to 8 mm. Microscopic examination showed that the tumor was located mainly in submucosa and muscularis propria of the stomach, composed of glands and cysts surrounded by bundles of smooth muscle tissue (Figure 1B). Epithelial cells lining the glands and cysts were columnar and flattened mucinous cells (Figure 1C). Cellular stratification was minimal and nuclei were basally located with only slight atypia. Mitotic activity was absent. Complete sectioning of this mass failed to demonstrate exocrine or endocrine pancreatic structures. Accumulations of inflammatory mononuclear cells were noted near the glands.

DISCUSSION

AM of the gastrointestinal tract is rare, most frequently observed in the duodenum, gall bladder and stomach. Most cases of gastric AM occur in adults with no symptom or have nonspecific gastrointestinal symptoms. Scare clinical manifestations such as localized peritonitis have been reported[6]. To the best of our knowledge, this is the first case of gastric AM presented with melana. We ascribe the hemorrhage to mucosal ulcer.

In spite of the availability of newer diagnostic techniques, including CT and endoscopic ultrasonography[7], it is still difficult to diagnose gastric AM before operation. Endoscopic examination cannot differentiate gastric AM from other extramucosal lesions, such as gastrointestinal stromal tumor, lipoma, neurilemmoma, hemangioma, gastrointestinal autonomic nerve tumor, carcinoid, lymphoma or even gastric carcinoma. In the majority of cases, endoscopic biopsies are superficial and fail to obtain representative tumor tissue. Therefore, frozen section is very helpful to establish the intraoperative diagnosis and to avoid unnecessary extensive operations. In the present case, two biopsies were not diagnostic, frozen section revealed the possibility of gastric AM.

Histologically, gastric AM should be differentiated from high-grade adenocarcinoma and gastritis cystica profunda. Gastric adenocarcinoma associated with gastric AM has been reported[8]. The common features that favor an adenocarcinoma are epithelial atypia, mitotic figures, and fibrous stromal reaction. Gastritis cystica profunda (GCP) is characterized by elongation of gastric foveola with hyperplasia and cystic dilatation of gastric glands, extending into the gastric submucosal layer. The absence of smooth muscle bundles around the cysts helps to differentiate GCP from AM.

Based on the similarities of epithelial component of AM to pancreatic ducts, several authors consider gastric AM as a variant of ectopic pancreas without exocrine or endocrine components[9,10]. Yamagiwa *et al*[11] divided ectopic pancreas into type Ⅰ (with all elements of normal pancreatic tissues), type Ⅱ (with pancreatic tissues but no islets), and type Ⅲ (with pancreatic ducts only). Gastric AM represents type Ⅲ. However, Takeyama *et al*[6] considered AM as a hamartoma based on the disordered proliferation of smooth muscle.

In summary, although gastric AM is rare, it should be always considered in the differential diagnosis of extramucosal gastric lesions. Despite the development of modern diagnostic techniques, its diagnosis remains challenging. If in doubt, frozen section can help to avoid unnecessary radical operation.

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LETTERS TO THE EDITOR

Hepatoprotective effects of antioxidants in chronic hepatitis C

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Abstract

We have read with interest the paper published in issue 2, volume 16 of *World Journal of Gastroenterology* 2010 by Nakamura *et al*, demonstrating that the antioxidant resveratrol (RVT) enhances hepatitis C virus (HCV) replication, consequently, they conclude that RVT is not a suitable antioxidant therapy for HCV chronic infection. The data raise some concern regarding the use of complementary and alternative medicine since the most frequent supplements taken by these patients are antioxidants or agents that may be beneficial for different chronic liver diseases. A recent study by Vidali *et al* on oxidative stress and steatosis in the progression of chronic hepatitis C concludes that oxidative stress and insulin resistance contribute to steatosis, thus accelerating the progression of fibrosis. We are particularly interested in investigating how the oxidative and nitrosative stress mechanisms are involved in the pathogenesis of different chronic liver diseases.

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Key words: Antioxidant; Chronic hepatitis C; Resveratrol; S-adenosyl-methionine; Nitric oxide; N-acetyl-cysteine

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TO THE EDITOR

We read with interest the paper by Nakamura *et al*[1] demonstrating that the antioxidant resveratrol (RVT) enhances the hepatitis C virus (HCV) replication, consequently, they conclude that RVT is not a suitable antioxidant therapy for HCV chronic infection. The data raise some concern regarding the use of complementary and alternative medicine since the most frequent supplements taken by these patients are antioxidants or agents that may be beneficial for different chronic liver diseases. A recent study by Vidali *et al*[2] on oxidative stress and steatosis in the progression of chronic hepatitis C (CHC) concludes that oxidative stress and insulin resistance contribute to steatosis, thus accelerating the progression of fibrosis. They speculate that therapeutic regimens including anti-oxidant agents could be of clinical relevance in CHC patients infected with genotype non-3, according their own results[2]. There is evidence that antioxidant therapies may ameliorate the necro-inflammatory activity in CHC patients[3].

We are particularly interested in investigating how the oxidative and nitrosative stress mechanisms are involved in the pathogenesis of different chronic liver diseases[4]. Based on this rationale, we would like to support the hypothesis that antioxidant compounds might be proven beneficial for acute and chronic liver diseases. According to ours and other author experience, nitric oxide (NO)

has deleterious consequences in the presence of reactive oxygen species, since peroxynitrites are formed. NO participates in the pathophysiology of viral (chronic hepatitis B and CHC) and autoimmune (primary biliary cirrhosis and autoimmune hepatitis) liver diseases[5,6], as well as in acute liver allograft rejection[7]. Moreover, NO contributes to liver ischemia-reperfusion injury and inhibition of inducible nitric oxide synthase (iNOS) shows beneficial effects[8]. Consistently, we found that N-acetyl-cysteine (NAC) modulates the expression of iNOS in human hepatocytes stimulated by proinflammatory cytokines[9]. The effect occurs by blocking the activation of the iNOS promoter, and is associated with modulation of NF-κB activity, a central transcription factor for induction of iNOS expression. The biological phenomenon might well be the basis of the therapeutic effects of NAC on chronic liver diseases different from those caused by acetaminophen intoxication.

Further insights into the hepatoprotective mechanisms of antioxidants might be learnt by analysing the glutathione precursor S-adenosyl-methionine (SAMe) actions. Administration of SAMe to patients with alcoholic liver cirrhosis showed beneficial effects[10]. Its possible mechanisms of action include: (1) acting as a methyl donor compound and contributing to restoration of mitochondrial glutathione content, which is necessary to counterbalance the oxidant environment in cirrhotic liver, and (2) attenuating the hepatic production of NO, through the modulation of nitric oxide synthase-2. SAMe exerts these effects by accelerating re-synthesis of inhibitor κB alpha and blunting the activation of nuclear factor κB, thereby reducing the transactivation of NO synthase-2 promoter[11].

Taking all these data together, abundant evidence suggests that antioxidants can effectively attenuate the oxidative and nitrosative stress in liver injury, ultimately improving inflammation and fibrosis progression. It is worth testing these drugs in future clinical trials including CHC patients, mainly those who present negative predictive factors of sustained virological response to standard antiviral regiments. However, controversies raise from the results of the study by Nakamura *et al*[1] promoting the possible need of investigations on the effects of different antioxidants on HCV replication before its use as a supplement in treatment of CHC patients.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
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The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

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Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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Organization as author

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

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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Conference proceedings

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

Conference paper

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Electronic journal (list all authors)

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *H. pylori*, *E coli*, *etc.*

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