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EDITORIAL

Gut flora and bacterial translocation in chronic liver disease

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Abstract

Increasing evidence suggests that derangement of gut flora is of substantial clinical relevance to patients with cirrhosis. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen, in particular, predispose to an increased potential for bacterial infection in this group. Recent studies suggest that, in addition to their role in the pathogenesis of overt infective episodes and the clinical consequences of sepsis, gut flora contributes to the pro-inflammatory state of cirrhosis even in the absence of overt infection. Furthermore, manipulation of gut flora to augment the intestinal content of lactic acid-type bacteria at the expense of other gut flora species with more pathogenic potential may favourably influence liver function in cirrhotic patients. Here we review current concepts of the various inter-relationships between gut flora, bacterial translocation, bacterial infection, pro-inflammatory cytokine production and liver function in this group.

Key words: Gut flora; Bacterial translocation; Cirrhosis

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INTRODUCTION

Disturbances of the microecology of the indigenous gut flora are prevalent in patients with chronic liver disease. Significantly increased viable counts of both Gram-positive and negative bacterial species have been recovered from faeces of cirrhotic patients[1]. A high prevalence of small intestinal bacterial overgrowth, in particular, has been demonstrated in this group[2-5]. This has been attributed, at least in part, to a decrease in small intestinal motility associated with increased adrenergic activity and portal hypertension[4-8]. Studies performed in experimental animals demonstrating important effects of gut flora on intestinal motility[9,10] suggest that the development of small intestinal bacterial overgrowth in the setting of intestinal stasis may further impair intestinal motility, thereby creating a self-perpetuating vicious cycle.

In addition to the range of symptoms and consequences of bacterial overgrowth *per se*[11], increasing evidences suggest that derangement of gut flora is of substantial clinical relevance to patients with cirrhosis. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen, in combination with failure of immune defence mechanisms to efficiently remove these translocating microorganisms, predispose to an increased potential for bacterial infection in this group[12]. Impaired immune defences include reduced opsonic activity due to low hepatic synthesis of complement, deranged function of macrophage Fc gamma receptors and reduced phagocytic and killing capacity of neutrophils[13-16]. Impairment of mucosal immunity may also be important. The high prevalence of associated malnutrition in cirrhotic patients[17] exacerbates the potential for infection to occur.

Recent studies suggest that, in addition to their role in the pathogenesis of overt infective episodes and the clinical consequences of sepsis, gut flora contributes to the pro-inflammatory state of cirrhosis even in the absence of overt infection[18]. Furthermore, manipulation of gut-flora using either a Gram-positive synbiotic (probiotic and fermentable fibre) regimen or fermentable fibre alone to augment the intestinal content of lactic acid-type bacteria at the expense of other gut flora species with more pathogenic potential may favourably influence liver function in cirrhotic patients[19]. Here we review current concepts of the various inter-relationships between gut flora, bacterial translocation, bacterial infection, pro-inflammatory cytokine production and liver function in this group.

BACTERIAL TRANSLOCATION IN CIRRHOSIS

Bacterial translocation is defined as the migration of bacteria from the intestinal lumen to mesenteric lymph nodes or other extra-intestinal sites[20]. Gram-negative

members of the Enterobactereaceae family (such as *Escherichia coli* and *Klebsiella spp*), enterococci and other streptococci species are the most effective at bacterial translocation to mesenteric lymph nodes[21]. These bacterial flora, the organisms most commonly implicated in community-acquired infective episodes in patients with cirrhosis[22], can translocate across even histologically normal intestinal mucosa[21,23]. Certain strains of *Escherichia coli* are especially efficient at translocation, possibly as a result of a greater ability to adhere to the intestinal mucosal surface[24]. While obligate anaerobic bacterial flora outnumber aerobic species by more than 100-fold, these flora only rarely translocate from the intestinal lumen[21]. Conversely, anaerobic strains limit the growth of other species with higher translocation potential and their selective elimination has been shown to promote translocation of such aerobic flora[25].

Most data in support of the occurrence of increased bacterial translocation in cirrhosis come from studies performed in experimental animals, in which bacterial translocation was defined by the presence of positive bacteriological cultures for gut flora in surgically-removed mesenteric lymph nodes[26]. These studies demonstrate that the prevalence of bacterial translocation to mesenteric lymph nodes is around 40% in cirrhotic rats with ascites and around 80% in such animals with spontaneous bacterial peritonitis (SBP)[27-31]. The concept of bacterial translocation predisposing to infection in experimental cirrhosis is further supported by data showing that bacteria isolated from mesenteric lymph nodes are genetically identical to strains causing SBP in the same animal[32].

Clinical studies of bacterial translocation in cirrhosis have been limited by the lack of non-invasive methods to detect its presence. Nonetheless, available evidence suggests that increased translocation of gut flora does occur in cirrhotic patients. A high rate of positive mesenteric lymph node cultures for enteric bacteria (30.8%) has been reported in patients with Child-Pugh class C cirrhosis undergoing liver transplantation or hepatic resection, with the incidence in the order of five times higher in this group than in Child-Pugh class A or B patients[33]. In another study, almost 20% of cirrhotic patients were found to have positive mesenteric lymph node cultures following partial hepatectomy, with bacteria responsible for instances of post-operative infection the same as those recovered from mesenteric lymph nodes in most cases[34].

The presence of bacterial DNA in serum and ascitic fluid has recently been proposed as a relatively non-invasive surrogate marker of bacterial translocation in the clinical setting[35]. Using such molecular techniques, bacterial translocation may be present in as many as one third of cirrhotic patients with portal hypertension and culture-negative ascites, with *Escherichia coli* the most frequently identified bacterial species[35]. The clinical relevance of the presence of bacterial DNA in culture-negative ascites has been suggested by the finding of increased local levels of pro-inflammatory cytokines[36], although the validity of this approach and the full clinical relevance of a positive result remain to be firmly established. Increased serum levels of lipopolysaccharide-binding protein, a phenomenon attributed to translocation of bacteria from the gut to the circulation, have been documented in a proportion of cirrhotic patients without overt infection and to predict the later development of severe bacterial infection in this group[37].

Pathogenesis of bacterial translocation in cirrhosis

Intestinal bacterial overgrowth Intestinal bacterial overgrowth is a major factor promoting bacterial translocation[38-41] and available evidence suggests that factors that promote intestinal bacterial overgrowth in particular, such as impaired intestinal motility, are important in facilitating bacterial translocation in cirrhosis. In experimental animals with cirrhosis, strategies to increase the intestinal transit rate, such as treatment with propranolol or cisapride, have been shown to reduce bacterial overgrowth and bacterial translocation[38,40,41]. In the clinical setting, cisapride has been shown to increase small intestinal motility and reduce bacterial overgrowth in patients with cirrhosis, with a trend towards a lower incidence of infection in treated patients[3,42].

Structural changes in intestinal mucosa Most clinical studies performed to investigate structural changes of intestinal mucosa in patients with cirrhosis have focussed on the small intestine. Shorter and thicker microvilli have been described. Morphologically intact tight junctions, which join together the apical poles of enterocytes and represent the first line of mucosal defence against paracellular absorption, have been reported in a small cohort of clinically stable cirrhotic patients with no prior history of infection with gut-derived bacteria[43]. Whether tight junctions are also intact in cirrhotic patients with a history of such infection and those with elevated nitric oxide levels may be more relevant, since nitric oxide, which is discussed in more detail below in relation to the clinical course of SBP, has been shown to reversibly dilate tight junctions in cultured intestinal epithelial cells[44]. Notably, dilatation of the intercellular space below tight junctions, the second line of defence against paracellular absorption, has been documented in patients with cirrhosis[43]. Mucosal alterations attributed to oxidative stress, including disturbed enterocyte mitochondrial function and increased lipid peroxidation of brush border membranes, have been reported in experimental animal models of cirrhosis[45], although the relevance of these changes to the pathogenesis of bacterial translocation is uncertain.

Thick-walled, dilated capillaries along with oedema of the lamina propria, fibromuscular proliferation, a reduced villus/crypt ratio and thickened muscularis mucosa in the small bowel have been found in cirrhotic patients with portal hypertension[46] and it has been proposed that an increased potential for bacterial translocation may exist in this setting[47]. However, in a study of cirrhotic patients with an elevated mean portal venous pressure in the order of 25 mm Hg undergoing liver transplantation, portal venous pressure was not significantly different in 8 cirrhotic patients with bacterial translocation and 71 with negative mesenteric lymph node culture results, implying that additional factors are required for bacterial translocation to occur in this group[33].

Luminal factors contributing to intestinal barrier function Other factors that contribute to the normal intestinal barrier against bacterial translocation (BT), including luminal factors, such as levels of bile acids, secretory immunoglobulin A, mucins, defensins, lysozyme and phopholipase A2, have been little studied in cirrhotic patients. Bile acids exert a trophic effect on intestinal mucosa[48] and inhibit intestinal bacterial overgrowth, especially of Gram-positive species[49]. An increased incidence of bacterial translocation in patients with obstructive jaundice has been reported[50]. However, whether the reduced intestinal luminal levels of bile acids that may be found in cirrhotic patients, as a consequence of reduced hepatic secretion and luminal deconjugation by overgrowth bacteria[12], contribute importantly to the potential for increased bacterial translocation in this group has not been established.

Intestinal immunity Little is known concerning the functional capacity of intestinal immunity in patients with cirrhosis and whether any disturbance of intestinal immune mechanisms contributes importantly to bacterial translocation, especially in the clinical setting. An increased number of intraepithelial lymphocytes with markedly impaired proliferative activity and capacity for production of interferon-γ have been reported in a murine model, with these changes correlating with increased bacterial translocation[51]. Whether this mechanism is important in the clinical situation is unknown. Notably, modest increases in small intestinal intraepithelial lymphocyte counts are found in patients with small intestinal bacterial overgrowth[52], raising the possibility that the increased levels found in cirrhotic animals may be explained on this basis. Whether the number or function of dendritic cells and other mononuclear cells in intestinal mucosa are deranged and contribute to the pathogenesis of bacterial translocation in patients with cirrhosis remains to be defined.

Anatomical site of bacterial translocation in cirrhosis

The anatomical site(s) from which bacterial translocation in cirrhosis occurs remains to be determined. Data in the non-cirrhotic setting suggest that the small intestine rather than the colon may be the major site of bacterial translocation. In particular, experimental studies involving inoculation of various regions of the gastrointestinal tract with equal quantities of *Escherichia coli* suggest that translocation occurs preferentially from the small bowel rather than the colon[53]. Similarly, in experimental animals with impaired intestinal motility, treatment with the prokinetic agent, cisapride, is associated with a reduced rate of bacterial translocation that parallels a reduction in jejunal but not caecal bacterial counts[3].

Intestinal permeability and its relation to bacterial translocation in cirrhosis

Functional studies have demonstrated that increased intestinal permeability, as reflected by dual sugar absorption tests or absorption of other test substances, occurs in patients with cirrhosis, especially in those with advanced liver disease[54-58]. Factors predisposing to this increased permeability in cirrhosis remain to be defined. In particular, any relationships with the structural changes of intestinal mucosa described above are uncertain. Enhanced intestinal permeability, likely via the paracellular route, has been reported in association with small intestinal bacterial overgrowth with Gram-negative gut flora in the non-cirrhotic setting[52], raising the possibility that this entity may at least contribute to the increased intestinal permeability in patients with cirrhosis. The relationship between intestinal permeability and the potential for bacterial translocation is uncertain, as increased permeability results have been found to correlate with a history of septic complications in some, but not all, reports.

GUT FLORA AND BACTERIAL INFECTION IN CIRRHOSIS

Bacterial infection, especially with intestinal-type flora, is a common complication in patients with cirrhosis[59-62]. Spontaneous bacterial peritonitis (SBP), urinary tract infection, respiratory tract sepsis (pneumonia and spontaneous bacterial empyema) and bacteraemia are the most frequent infective complications occurring in this group. The incidence of infection with Gram-positive cocci, in particular, has increased in recent years, with such flora now the most frequent isolates in hospitalised cirrhotic patients with nosocomial infection, especially those admitted to intensive care units, presumably due to the high rate of invasive procedures, including placement of indwelling vascular and bladder catheters, performed in this group[62]. Recent data suggest that between 15% and 35% of cirrhotic patients admitted to hospital develop nosocomial bacterial infection, substantially higher than the rate of hospital-acquired infection in the order of 5% to 7% in the general hospital setting[63].

Complicating infection may have severe adverse clinical consequences in cirrhotic patients. The associated pro-inflammatory response exacerbates hepatic dysfunction, encephalopathy and the haemodynamic disturbances that underlie the development of portal hypertension and hepatorenal syndrome[60,64,65]. Increasing evidence suggests that bacterial infection is a trigger for variceal haemorrhage in patients with cirrhosis[66,67], possibly as a consequence of both activation of hepatic stellate cells, leading to increased intrahepatic vascular resistance, and prostacyclin-related inhibition of platelet aggregation[66]. Conversely, variceal haemorrhage predisposes cirrhotic patients to bacterial infection with gut-derived flora[68-73], setting up a vicious cycle between gastrointestinal bleeding and infection in this group. This is a phenomenon of substantial clinical importance, as complicating infection is independently associated with early mortality in bleeding cirrhotic patients[69,70,72]. Worsening coagulopathy, due to the consumption of clotting factors by the extrinsic coagulation pathway[74] and the production of endogenous heparinoids[75], occurs in patients with cirrhosis and complicating bacterial infection. Sepsis is a common cause of death in cirrhotic patients[60,61,76-78]. The mortality rate associated with bacterial infection in this group is more than twenty times higher than in the general population[79].

SPONTANEOUS BACTERIAL PERITONITIS(SBP)

SBP, an infection of ascitic fluid typically with a single bacterial species in the absence of any other primary intra-abdominal source, is the most characteristic and serious infection occurring in patients with cirrhosis. Prospective studies suggest that SBP, including culture-negative and non-neutrocytic cases, is present in up to 23% of all cirrhotic patients with ascites admitted to hospital[80]. Gram-negative gut flora, especially *Escherichia coli* and *Klebsiella* species, are isolated in approximately 70% of culture-positive cases of community-acquired SBP. Aerobic Gram-positive bacteria belonging to *Streptococcus* and *Staphylococcus* spp constitute most of the remaining isolates. Pathogens belonging to *Aeromonas, Plesimonas, Listeria, Salmonella* and *Neisseria* spp are occasionally responsible. In keeping with their reduced potential for translocation from the intestine discussed above, obligate anaerobes are isolated in fewer than 5% of cases[81]. In hospitalised cirrhotic patients with nosocomial SBP, Gram-positive pathogens are predominant, accounting for over 70% of isolates, with methicillin-resistant *Staphylococcus aureus* accounting for nearly 25% of cases in one recent series[82].

The overall likelihood of a cirrhotic patient with ascites developing SBP at one year is in the order of 10%[80]. Clinical studies have identified several sub-groups of patients with cirrhotic ascites at particularly high risk for SBP. Over 70% of cases occur in those classified as Child-Pugh C[83]. An increased prevalence of SBP has been reported in patients with small intestinal bacterial overgrowth compared to their non-overgrowth counterparts, presumably due to the increased propensity for bacterial translocation[84]. Gastrointestinal haemorrhage is another important precipitant of SBP in this group. Bacteraemia and/or SBP, typically with Gram-negative enteric-type flora, develop within 48 hours of gastrointestinal haemorrhage in nearly 50% of Child-Pugh C patients with ascites[85]. The likelihood of SBP is related to the functional activity of Kupffer cells, which is impaired in patients with advanced liver disease[86]. However, the most powerful predictive factor identified in several series is an ascitic fluid total protein level $\leqslant$10 g/L, which reflects a low complement protein C3 concentration and, hence, opsonisation capacity. Patients with such low levels are at a six- to ten-fold increased risk of a first episode of SBP compared to cirrhotic counterparts with ascitic fluid total protein levels > 10 g/L[87,88].

CLINICAL COURSE

Despite a trend towards earlier diagnosis and commencement of antibiotic treatment, along with overall improvements in the general medical care of cirrhotic patients, the in-hospital mortality rate associated with SBP remains 30-50%[80]. This is largely related to underlying hepatic decompensation and a high prevalence of complicating hepatorenal syndrome. The latter occurs in approximately 30% of patients with SBP, predominantly in those with pre-existing renal impairment, and is progressive despite cure of infection in half of these cases[89]. Ascitic levels of nitric oxide, which are significantly increased in cirrhotic patients with SBP and persist for over two weeks despite appropriate antibiotic treatment[90,91], independently predict renal impairment in this setting[92]. Use of nephrotoxic antibiotics adds to the risk of renal failure in this setting[93]. The development of renal failure is the most important predictor of in-hospital mortality associated with SBP. This is in the order of 50% in those with complicating renal failure compared to only 6% in those without[89]. Albumin infusion improves systemic haemodynamics, prevents renal failure and improves survival compared to antibiotic treatment alone in patients with SBP. Beneficial effects are related to reduction in arterial vasodilatation and improved cardiac function[94]. Other correlates of poor outcome include increased ascitic levels of the pro-inflammatory cytokines, interleukin-6 and tumor necrosis factor-α (TNF-α)[95]. A recent report suggests a higher mortality rate in patients with nosocomial infection with staphylococcal species compared to that in patients with community-acquired SBP[82]. A trend towards a higher mortality rate when infection is with encapsulated strains of *Escherichia coli* associated with tissue invasiveness has also been reported[96].

The medium term prognosis for cirrhotic patients who have recovered from an episode of SBP is similarly poor, with a mortality rate at one year in the order of 30-80%[80]. Approximately 20% of patients who die within one year of an episode of SBP succumb to a further episode of spontaneous peritoneal infection, the remainder dying of causes, such as variceal haemorrhage, hepatorenal syndrome or hepatocellular carcinoma[97-99]. The risk of SBP recurrence within one year ranges from 40-70% and, as for an initial episode, is influenced mainly by the degree of underlying liver dysfunction and the ascitic fluid total protein level[83,98,99]. Survivors of an episode of SBP should be evaluated for liver transplantation in view of the high risk of recurrence and poor overall prognosis.

PROPHYLAXIS

Antibiotic prophylaxis against SBP may be beneficial in three high risk groups of cirrhotic patients, namely those who have survived a previous episode, those with low ascitic fluid total protein levels and those presenting with gastrointestinal haemorrhage[67-71,73,100-103]. Most prophylaxis studies have aimed to reduce or eradicate aerobic Gram-negative bacilli from the intestine using norfloxacin, a poorly absorbed quinolone with activity against these flora. The importance of antibiotic prophylaxis in patients with cirrhosis presenting with variceal haemorrhage is increasingly recognised. Treatment with norfloxacin, 400 mg twice daily either orally or via a nasogastric tube for seven days commencing immediately after emergency endoscopy, was associated with a significantly reduced in-hospital incidence of bacteraemia and/or SBP, especially with aerobic Gram-negative flora, compared to patients receiving no prophylactic antibiotics (3% *vs* 17%)[68]. Ciprofloxacin, 500 mg twice daily either orally or via nasogastric tube for 7 d immediately following endoscopy, has similarly been shown to significantly reduce the incidences of bacteraemia, SBP and urinary tract infection compared to placebo (0% *vs* 23%, 3% *vs*

13% and 5% *vs* 18%, respectively)[103]. Implementation of treatment protocols that incorporate antibiotic prophylaxis led to a fall in post-variceal bacterial infection rates from 38% to 14% over the past two decades in a large French centre[69]. Antibiotic prophylaxis has been shown to reduce the incidence of not only bacterial infection, but also early re-bleeding following variceal haemorrhage, especially in Child-Pugh class C patients, those requiring ventilatory support and those initially treated with balloon tamponade[67,70,71,73].

Several cost analysis studies have shown that prophylactic treatment with norfloxacin in each of these high risk groups is cost effective, as a consequence of the reduced incidence of SBP and its associated resource utilisation[67,104,105]. Short-term prophylaxis with trimethoprim-sulphamethoxazole is also cost-effective, especially in those at high risk for SBP[105]. Most studies report a reduction in overall mortality associated with prophylactic antibiotic treatment, although a statistically significant survival benefit has been more difficult to demonstrate. This is not surprising, as the likelihood of dying as a result of progressive liver failure, variceal haemorrhage, hepatocellular carcinoma or other causes is unaffected by the use of prophylactic antibiotics. More recently, a meta-analysis of 13 randomized controlled trials has suggested that antibiotic prophylaxis of hospitalized cirrhotic in-patients is efficacious in reducing the relative risk of in-hospital death (relative risk of dying 0.70), irrespective of underlying risk factors[107]. In addition, a meta-analysis of 534 cirrhotic patients with gastrointestinal bleeding has shown that short-term antibiotic prophylaxis significantly increases short-term survival rates by a mean 9% in this setting[107].

A particular concern, especially with long-term antibiotic prophylaxis, is the potential for the development of infection with antibiotic-resistant bacteria. Initial studies of norfloxacin prophylaxis against SBP reported a low incidence of infection with quinolone-resistant Gram-negative bacterial species. More recent studies, however, suggest an increased prevalence of infection with such microorganisms. Over 20% of cases of SBP due to *Escherichia coli* were with a norfloxacin-resistant strain in a recent series from Spain[108], while 50% of cases of culture-positive SBP in patients receiving long-term norfloxacin prophylaxis and 16% of instances in patients not treated with this drug were caused by norfloxacin-resistant Gram-negative bacteria in another Spanish report[62]. A high rate of SBP due to trimethoprim-sulphamethoxazole-resistant Gram-negative flora in excess of 40% was also documented in norfloxacin-treated patients. An increased proportion of infections with Gram-positive bacterial species has also been reported in this setting, including instances of severe hospital-acquired staphylococcal infections[109]. An increased incidence of carriage of methicillin-resistant *Staphylococcus aureus* has also been documented in cirrhotic patients treated with norfloxacin prophylaxis[110].

Such experiences highlight the need for non-antibiotic-based strategies to prevent intestinal bacterial overgrowth, bacterial translocation and SBP in patients with cirrhosis. As alluded to earlier, a six-month trial of the pro-motility agent, cisapride, was associated with improved small intestinal motility, reversal of bacterial overgrowth and a tendency towards a reduced incidence of infection with gut-derived flora in a cohort of cirrhotic patients[3,42]. Unfortunately, cisapride has been withdrawn from use in some countries because of potential for cardiac arrhythmia. Alternatively, treatment with either symbiotics (in the form of four species of lactic acid bacteria and fermentable fibre) or fermentable fibre alone has recently been shown to significantly reduce viable counts of potentially pathogenic Gram-positive and Gram-negative gut flora in patients with cirrhosis[19]. In another study, a lower rate of post-operative bacterial infection was documented in liver transplant recipients treated with an early enteral supply of a synbiotic regimen that included *Lactobacillus plantarum* and fermentable fibre than in patients receiving selective intestinal decontamination[111]. A follow-up randomised double-blind trial in liver transplant recipients showed that early enteral nutrition supplemented with a mixture of lactic acid bacteria and fermentable fibre significantly reduced the incidence of post-operative bacterial infection compared to supplementation with fermentable fibre alone[112]. Based on these reports, the possible use of synbiotics for prophylaxis against SBP in cirrhotic patients warrants investigation. Conversely, probiotic treatment with a lactobacillus strain without additional fermentable fibre was ineffective in preventing bacterial translocation and SBP in rats with experimental cirrhosis, despite successful intestinal colonisation[113].

GUT FLORA AND PRO-INFLAMMATORY STATE OF CIRRHOSIS IN ABSENCE OF OVERT INFECTION

Pro-inflammatory cytokines, such as TNF-α, have been shown to be critically involved in the development and/or exacerbation of liver injury in animal models[115,116]. Activation of macrophages by endotoxin, a cell wall component of Gram-negative bacteria, plays a key role in the over-production of TNF- α and liver injury in these settings[117,118]. Increased translocation from the gut lumen and reduced hepatic clearance have each been proposed to predispose to endotoxaemia in this situation[119,120]. In the clinical setting, increased circulating levels of both endotoxin and pro-inflammatory cytokines have been documented in patients with chronic liver disease, even in the absence of overt infection[120-125]. Based on experiences in experimental animals, it has, until recently, been assumed that endotoxaemia causes the raised pro-inflammatory cytokine levels in this group[121,126-128]. However, a significant correlation between circulating endotoxin and pro-inflammatory cytokine levels has generally not been show n[18,120,121,124,126], raising the possibility that, unlike in animal models, stimuli other than endotoxin may be important.

Recent analyses of the expression of Toll-like receptors (TLRs) in patients with cirrhosis have helped to clarify this issue. These receptors are the human homologues of the Drosophila Toll protein and comprise a family of at least ten transmembrane receptor proteins, the intracellular domains of which show distinct sequence homology with the interleukin-1 receptor. TLRs play a critical role in the

induction of innate immunity to microbial pathogens via recognition of conserved molecular patterns. In particular, it is known that TLR4, in association with CD14 and MD-2, is responsible for signal transduction leading to TNF-α production in response to endotoxin. In contrast, TLR2 is required for signaling in response to a number of Gram-positive microbial stimuli, including whole bacteria and cell wall components, such as peptidoglycan and lipoteichoic acid[129-131]. Indeed, significantly increased expression on peripheral blood mononuclear cells (PBMCs) of TLR2 but not of TLR4 has recently been demonstrated in patients with cirrhosis but no overt infection[18]. Furthermore, PBMC expression of TLR2 but not that of TLR4 was shown to correlate significantly with circulating levels of both TNF-α and anti-inflammatory soluble TNF receptors[18]. These novel findings suggest that signaling via TLR2 but not TLR4 contributes to the increased circulating levels of TNF-α levels found in cirrhosis and imply, contrary to previous assumptions, an important role for Gram-positive microbial stimuli rather than endotoxin in this process. This contention is supported by *in vitro* PBMC stimulation data consistent with presensitisation by Gram-positive microbial stimuli but not endotoxin *in vivo*. In addition, supplementation of cirrhotic patients with a synbiotic regimen including four Gram-positive gut flora and fermentable fibre led to further increases in PBMC expression of TLR2 and circulating TNF-α levels in most cases, suggesting that Gram-positive stimuli derived from the gut, in particular, may be important in promoting the increased circulating levels of both pro-inflammatory and anti-inflammatory TNF-related molecules found in patients with cirrhosis[18].

GUT FLORA AND HEPATIC FUNCTION IN CIRRHOSIS

Reversal of minimal hepatic encephalopathy has been reported in 50% of predominantly Child-Pugh class B or C cirrhotic patients treated with a lactic acid bacteria-based synbiotic regimen for 30 d, an efficacy comparable to that of treatment with lactulose[19]. In addition, the Child-Pugh class improves in nearly 50% of initially Child-Pugh class B or C synbiotic-treated patients, a proportion significantly higher than that in placebo-treated counterparts (8%). Treatment is associated with a significant increase in the faecal content of lactobacilli at the expense of potentially pathogenic Gram-positive and negative bacterial species. Bacterial overgrowth is also reversed and improvements in minimal hepatic encephalopathy and the Child-Pugh class occurs in 50% and 29%, respectively, of cirrhotic patients treated with fermentable fibre alone. Both in synbiotic- and fermentable fibre alone-treated patients, improvement in the Child-Pugh class occurs as a result of significant improvements in the serum bilirubin and albumin levels and in prothrombin activity. Significantly reduced hepatic necro-inflammatory activity, as reflected by serial ALT levels, is documented in both groups. Improvement in clearance of indocyanine green has also been reported in cirrhotic patients treated with synbiotics[132]. These findings are in keeping with reported experiences in experimental animals' suggesting that oral supplementation with Gram-positive probiotics can protect against hepatocellular damage. In particular, rats fed lactobacilli have been shown to be protected against alcohol-induced liver damage[133], while studies of probiotic use in a murine model of non-alcoholic fatty liver disease have found reductions in the intra-hepatic expression of various molecular markers of inflammation, including nuclear factor kappa-B and TNF-α[134,135].

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EDITORIAL

Hepatobiliary and pancreatic disorders in celiac disease

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Abstract

A variety of hepatic and biliary tract disorders may complicate the clinical course of celiac disease. Some of these have been hypothesized to share common genetic factors or have a common immunopathogenesis, such as primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune forms of hepatitis or cholangitis. Other hepatic changes in celiac disease may be associated with malnutrition resulting from impaired nutrient absorption, including hepatic steatosis. In addition, celiac disease may be associated with rare hepatic complications, such as hepatic T-cell lymphoma. Finally, pancreatic exocrine function may be impaired in celiac disease and represent a cause of treatment failure.

Key words: Celiac disease; Liver disease; Cholangitis; Autoimmune hepatitis; Hepatic vein obstruction; Primary biliary cirrhosis

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INTRODUCTION

A number of hepatobiliary and pancreatic disorders occur in celiac disease, a genetically-based small intestinal disorder that resolves with the complete restriction of dietary gluten[1]. Almost 3 decades ago, liver changes in celiac disease were first recognized by Hagander *et al*[2]. Later, Dickey *et al*[3] have confirmed these findings in a prospective evaluation of celiac disease patients and extended observations to results of gluten-free diet therapy. In some, these liver test changes are entirely reversible following administration of a gluten-free diet, while in others, clinically significant liver disease is not amenable to diet treatment alone[3]. Now, almost a decade after this report, recognition of celiac disease has been substantively improved, in part, a result of more modern serological assays for screening[4], the detection of tissue transglutaminase (tTG) as an autoantigen in celiac disease[5] and the increasingly widespread serological use of tTG ELISA to screen for celiac disease[6]. As a result of improved recognition of celiac disease, even more precise estimates of the overall disease burden related to hepatobiliary tract and pancreatic disorders will emerge.

In patients with unexplained elevations of liver enzymes, several studies using serological screening methods have estimated that almost 10% will prove to have celiac disease[7,8]. For example, Volta *et al*[7] examined endomysial and gliadin antibodies in 55 patients with elevations of liver chemistry tests in the absence of a known cause. Five patients had positive serological studies and small intestinal biopsies showed changes of celiac disease that responded to a gluten-free diet. Liver biopsies done in some patients showed a nonspecific inflammatory process and liver chemistry tests normalized with a gluten-free diet. Bardella *et al*[8] screened 140 patients with chronically elevated transaminase values for gliadin and endomysial antibodies; of these, 13 were seropositive. After 1 year on a gluten-free diet, 12 patients had normalization of liver enzyme tests.

In patients with known celiac disease, abnormal liver enzyme tests also occur[2,9-11]. Hagander *et al*[2] described elevated liver enzymes in 30 of 75 (40%) patients, while Bonamico *et al*[9] showed increased levels in 39 of 65 (60%) children, and Jacobsen *et al*[10] documented almost 50% with increased liver enzymes. In some, liver biopsy showed a nonspecific inflammatory process, although a more specific "chronic active hepatitis" was detected in 5 of 37 (13.5%) patients. Bardella *et al*[11] evaluated 158 consecutive adults with celiac disease and showed that 42% had abnormal liver enzyme levels. A gluten-free diet for 1 to 10 years resulted in complete normalization of liver chemistry tests in 95% patients.

In celiac disease, persistently abnormal liver chemistry tests may reflect the presence of a clinically occult hepatobiliary tract disorder with a possibly common immunopathogenesis. Specific examples of immune-mediated disorders include primary biliary cirrhosis, primary (lymphocytic, autoimmune) sclerosing cholangitis or autoimmune hepatitis. Alternatively, in some, a common genetically-based disorder, including altered control of small intestinal iron absorption resulting in a concomitant iron overload disorder, may be present, such as hemochromatosis. In addition, chronic changes

in liver chemistry tests may reflect a direct effect of the celiac disease. For example, impaired absorption and resultant malnutrition may lead to deposition of fat in the liver, related, in part, to reduced fat mobilization from hepatocytes. Indeed, massive hepatic steatosis has occasionally been reported in celiac disease. Finally, but very rarely, patients may develop a specific complication of celiac disease that involves the liver, such as a T-cell form of lymphoma.

HEPATOBILIARY TRACT DISEASES

Primary biliary cirrhosis

In 1978, Logan *et al*[12] described the first cases of primary biliary cirrhosis with celiac disease. Later, numerous additional cases have been reported[13-16]. In both disorders, other conditions having an immunological basis have been described, including diabetes and thyroiditis[16-19]. In addition, co-existence of primary biliary cirrhosis and celiac disease has not only been reported in Europe and the Americas, but also in migrants from South Asia[20] and the Coast Salish, an aboriginal population inhabiting the west coast of Canada thought to be of Asian descent[16]. To date, however, a definitive genetic predisposition or specific immunological alteration has not been clearly identified. Loss of weight, malabsorption, osteopenic bone disease, steatorrhea and elevated alkaline phosphatase activities are common features of both diseases, so that early in their coexistence, celiac disease or primary biliary cirrhosis may not be easily appreciated. In patients reported with both disorders, regardless of geographical origin or race, restriction of dietary gluten may have improved the diarrhea, but abnormal liver chemistry tests were usually not significantly altered with a gluten-free diet.

Some more recent studies have explored serological testing in primary biliary cirrhosis or celiac disease. Kingham and Parker[21] used a patient registry in the United Kingdom and defined the prevalence of primary biliary cirrhosis in 143 celiac patients as 3%, while the prevalence of celiac disease in 67 primary biliary cirrhosis patients was 6%. As a result, screening with antimitochrondrial antibodies in celiac disease was recommended, while in primary biliary cirrhosis, serological screening with gliadin antibodies or small intestinal biopsy was suggested. Dickey *et al*[22] found similar findings of 7% (4/57) primary biliary cirrhosis patients based on initial evaluation using endomysial antibodies (11% positive), followed by later duodenal biopsy confirmation. Despite 12 to 24 mo on gluten-free diets, however, improvement in liver chemistry tests was not detected even though endomysial antibodies disappeared. Using Danish and Swedish registry data based on over 8 000 patients with celiac disease, Sorensen *et al*[23] also suggested an increased risk of primary biliary cirrhosis. Using stored sera from 378 Canadian patients with primary biliary cirrhosis, Gillett *et al*[24] found that screening for IgA antibodies to endomysium and primary biliary cirrhosis were both positive in 10 (2.6%) patients and 5 patients had small intestinal biopsies confirming celiac disease. Intertestingly, however, another 44 primary biliary cirrhosis patients had raised IgA tissue transglutaminase antibodies but were negative for IgA endomysium antibody. In 255 patients with autoimmune cholestatic liver disorders, including 173 with primary biliary cirrhosis, Volta *et al*[25] found 9 with celiac disease (including 7 in those with primary biliary cirrhosis, 4%). In some recent studies, however, the importance of biopsy confirmation in patients with primary biliary cirrhosis has been demonstrated in sero-positive patients as false-positive IgA or IgG tTG antibodies may occur in primary biliary cirrhosis[26,27].

In a recent study using a general practice longitudinal database from the United Kingdom[28], an overall 3-fold risk of primary biliary cirrhosis was demonstrated in 4 732 patients diagnosed with celiac disease as compared with 23 620 age- and sex-matched controls.

Primary sclerosing cholangitis

Primary sclerosing cholangitis was first found to be associated with celiac disease in 1988 in 3 patients with diarrhea and steatorrhea[29]. Two also had concomitant "ulcerative colitis" (one with "inactive" quiescent disease and one with "mild" or "minimal change" colonic disease), a disorder known to be associated with primary sclerosing cholangitis. Although hepatobiliary tract changes were defined by cholangiography and liver biopsy, these did not respond to a gluten-free diet. Later, other cases were reported[10,25,30]. In one, the predominant lymphocytic nature of the portal inflammatory process was emphasized with increased intra-epithelial lymphocytes in biliary ductal epithelium[30], an observation also noted in gastric and colonic epithelium of celiac patients[31,33]. To date, despite some case report data[33], it has been difficult to show good evidence for a response of the hepatobiliary tract disease to a gluten-free diet. This may, in part, reflect sampling difficulties associated with liver biopsy as well as the response or lack of response of relatively non-specific liver chemistry test markers of cholestasis (e.g., serum alkaline phosphatase). Indeed, the origin of alkaline phosphatase activities measured in serum include the hepatobiliary tract and other tissues that may be substantially altered in celiac disease (i.e., bone and the intestine); conceivably all might be improved with a gluten-free diet.

Autoimmune hepatitis and cholangitis

This has been evaluated in only a limited numbers of case reports and survey studies. Unfortunately, many appeared before hepatitis C testing[13,35]. Jacobsen *et al*[10] performed liver biopsies in 37 of 171 celiac patients and found changes of "chronic active hepatitis" in 5 (2.3%) patients. Using antibodies to endomysium and gliadin, Volta *et al*[36] surveyed 157 patients with type 1 autoimmune hepatitis and 24 with type 2 autoimmune hepatitis for celiac disease. They found that 8 of these 181 (4%) patients were positive for endomysial antibodies, including 6 (4%) with type 1 disease and 2 (8%) with type 2 disease. Five of the 8 patients had a duodenal biopsy, most being asymptomatic, and all showed changes of subtotal villous atrophy, consistent with untreated celiac disease. The effects of steroid with or without azothioprine treatment on the underlying small intestinal histological changes were considered and also may have masked intestinal symptoms. Unfortunately, in this study, the effects of gluten-free

diet administration on the hepatic and intestinal changes were not reported. Recently, Villalta *et al*[37] evaluated 47 consecutive patients with autoimmune hepatitis, including 39 with type 1 disease and 8 with type 2 disease. Anti-IgA tissue transglutaminase and endomysial antibodies were positive in 3 (6.4%) patients and small intestinal biopsies confirmed the presence of the celiac disease histological changes[37].

Finally, celiac disease and other types of autoimmune liver and biliary tract disease may coexist. A case report of autoimmune cholangitis[38], a cholestatic liver disorder with biochemical evidence of cholestasis, histological evidence of inflammatory bile duct damage and an absence of anti-mitochondrial antibodies, was has been described in a patient with celiac disease. Interestingly, this patient's small intestinal biopsies were reported to be normal without a gluten-free diet while being treated with steroids and azathioprine. In an another case, Sedlack *et al*[39] reported an improvement in hepatic biochemistries without use of immunosuppressive agents.

HEMOCHROMATOSIS OR IRON OVERLOAD LIVER DISEASE

Celiac disease has been associated with hemochromatosis, which is not surprising, since both are relatively common disorders based on a common Celtic ancestry, so any association could be coincidental[40-42]. Iron absorption largely occurs in the proximal duodenum, the site most often histologically altered in celiac disease. Indeed, "isolated" iron deficiency with anemia may be the initial clinical manifestation of clinically occult celiac disease. In contrast, in iron overload liver disease, inappropriate iron absorption from the proximal small intestine occurs as body iron stores are markedly increased. In one of these early case reports, treatment of celiac disease and improvement in the pathological small intestinal changes led to worsening liver chemistry test values and recognition of occult iron overload liver disease (C282Y-negative), presumably related to improved intestinal uptake of dietary iron[41]. Another similar case of C282Y-positive hemochromatosis presented with diarrhea, positive anti-gliadin and endomysial antibodies. Subsequent small bowel biopsies showed villous atrophy[42]. Interestingly, in this latter case, phlebotomy therapy had to be terminated early because of an unexpectedly rapid fall in the serum ferritin measurement. A genetically-based linkage was also suggested since both diseases are associated with the HLA-region on chromosome 6. Later investigations have sought to resolve this possible relationship. Butterworth *et al*[43] observed that HFE (hemochromatosis susceptibility gene) locus mutations are common in celiac disease patients from the United Kingdom and may be important in protecting the celiac from iron deficiency, while others suggested that the significance of these observations may be controversial[44]. More recent studies in an Italian population with untreated celiac disease found that HFE mutations failed to protect against the development of iron deficiency[45]. Interestingly, in a recent case study of a patient with homozygous C282Y and celiac disease[46], reduced expression of the divalent metal transporter 1 (DMT1) was observed, but not ferroportin 1 (FP1) or transferrin receptor 1 (TfR1).

OTHER LIVER DISORDERS IN CELIAC DISEASE

Hepatic steatosis

Common causes of hepatic steatosis include alcohol-induced steatosis, diabetes mellitus, NASH syndromes and some forms of drug therapy, including corticosteroids. In some countries, dietary protein deficiency and kwashiorkor are important causes. Intestinal malabsorption is often associated with hepatic steatosis in patients with a prior jejunoileal bypass procedure for morbid obesity[47,48] and, sometimes, in those with inflammatory bowel disease, particularly after extensive intestinal resections[49]. Because celiac disease is now frequently recognized in a clinically occult form before manifestations of marked nutrient depletion are detected, hepatic steatosis is probably less common than in other intestinal diseases.

Several cases of fatty infiltration of the liver, often massive, have been described in adults with celiac disease[50-53]. Presumably, lesser degrees of hepatic fat deposition may occur. Most often if massive steatosis is evident, elevated transaminase and alkaline phosphatase activities have been documented along with alterations in coagulation. However, in most, clinical and biochemical changes attributed to the hepatic steatosis were improved with a gluten-free diet. In a patient with massive hepatic steatosis[52], a gluten-free diet for about 1 year also resulted in histological improvement in the fatty changes detected in the liver.

The mechanisms involved in fat deposition in the liver are not defined. Interestingly, after jejunoileal bypass, reduced serum levels of some essential and nonessential amino acids may be observed[47,48]. In addition, changes in serum amino acids have been recorded in patients with starvation-associated kwashiorkor[54,55]. Based on these nutritional disorders, it has been suggested that malabsorption in celiac disease might lead to chronic deficiency of a lipotropic factor (e.g., choline), with an associated pyridoxine deficiency, hepatic steatosis might occur[52]. Further studies are needed to define the precise pathogenetic mechanism or mechanisms for fatty liver in celiac disease.

Gallstone disease

Several studies have focused on gallbladder function in celiac patients. In some studies, slow emptying of the gallbladder has been documented[56,57], along with impaired contraction response to fat[56]. Studies of enteric endocrine cells showed significant quantitative changes in celiac patients, including complete absence of mucosal secretin cells[59]. In addition, studies with test meals have suggested impaired secretion of cholecystokinin in patients with celiac disease[59] or, possibly, impaired gallbladder responsiveness to cholecystokinin[56].

In spite of these physiological alterations, there does not appear to be a significant predisposition to gallstones in celiac disease. Only 9 of 350 patients had a cholecystectomy for gallstone disease[60]. However, in a

survey of elderly celiacs initially diagnosed after the age of 60 years, 6 of 30 (20%) had gallstone disease[61].

Hepatic vein obstruction

Although mesenteric vascular ischemia[62] and vasculitis[63-66] have been described in celiac disease, there are also reports of a unusual Budd-Chiari-like syndrome among celiac children from North Africa, particularly Tunisian and Algeria[67,68]. Hepatic vein obstruction has also been documented in 3 adults[69]. Deficiencies in protein C and antithrombin III are detected, and malabsorption of vitamin K in celiac disease has been proposed to cause transient protein C or protein S deficiencies. Further studies are needed to identify possible factors, either dietary or environmental agents, that may be important. More recently, a celiac patient with a Budd-Chiari syndrome associated with membranous obstruction of the inferior vena cava treated successfully with percutaneous balloon angioplasty has been reported[70].

Hepatic malignancies

While hepatocellular cancer has been reported in 1 patient, cirrhosis was also present[71]. Occasionally, the liver may be involved with lymphoma, the most frequently detected malignant disorder in celiac disease patients[72]. In some patients with celiac disease, lymphomatous deposits have been detected in the liver, presumably as metastatic lesions[71]. For example, lymphoma in the liver is apparently secondary to jejunal lymphoma, complicating celiac disease[71]. In general, involvement of the liver in celiac disease patients with lymphoma is limited and overshadowed by the clinical course of the intestinal disease. However, a fulminant cholestatic syndrome has been described in a celiac disease patient, resulting in hepatic failure[73]. Later investigations have shown widespread hepatic involvement with an unusual lymphoid neoplasm classified as a hepatosplenic lymphoma, a rare type of peripheral T-cell lymphoma with rearrangement of the gamma-delta T-cell receptor[74,75].

Liver failure

In patients with severe liver failure from a variety of causes in celiac disease, dietary treatment reverses hepatic dysfunction, even in patients with consideration for possible liver transplantation[76].

PANCREATIC DISEASE

While celiac disease is associated with insulin-dependent diabetes[77], pancreatic exocrine insufficiency and celiac disease have only occasionally been recorded[78-84]. Pancreatic calcification is most often associated with chronic or persisting pancreatic inflammation which is usually due to excessive consumption of alcoholic beverages. Atrophy, fibrosis and altered pancreatic function have been observed in experimental animals treated with diets deficient in protein, in adults with protein-energy malnutrition, in children with kwashiorkor and in some early autopsy studies of patients with celiac disease. In addition, pancreatic calcification has been reported with chronic protein malnutrition in the Indian subcontinent and in some African countries. Finally, a patient with celiac disease and pancreatitis with calcification has been described[83].

Although the frequency of pancreatic disease in celiac patients is not known, impaired pancreatic function occurs and may be a cause of persistently impaired nutrient assimilation and malnutrition. It has been estimated that over 20% of children with celiac disease have defective exocrine pancreatic function[85]. This may be related to several factors. Impaired secretion and/or release of pancreatic stimulating hormones from the diseased proximal small intestine may be important[60]. Immunohistochemical studies have demonstrated alterations in enteric endocrine cells, and in biopsies from patients with untreated celiac disease, an absence of secretin cells has been reported[59]. Studies with test meals in celiac patients have suggested impaired secretion of cholecystokinin-pancreozymin resulting in reduced pancreatic exocrine cell stimulation[81]. In addition, a deficiency of amino acids may result from impaired small intestinal amino acid uptake, leading to a reduction in precursors for pancreatic enzyme synthesis[55,80]. Also, protein malnutrition may lead to structural changes in the pancreas, including atrophy of acinar cells and pancreatic fibrosis[55], resulting in impaired pancreatic exocrine function. In a more recent study[86], pancreatic enzyme measurements were reduced with mucosal atrophy and could be inversely correlated with the degree of intestinal damage.

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EDITORIAL

Exacerbation of inflammatory bowel disease by nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors: Fact or fiction?

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Abstract

The existence of a possible link between inflammatory bowel disease (IBD) and nonsteroidal anti-inflammatory drugs (NSAIDs) has been repeatedly suggested. Recently, a few studies have addressed the issue of a possible, similar effect by selective cyclooxygenase-2 inhibitors (COXIBs). The present article reviews the available scientific evidence for this controversial subject.

Key words: COX-2 inhibitor; Inflammatory bowel disease; Non-steroidal anti-inflammatory drugs

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The use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with the onset of inflammatory bowel disease (IBD) or with a clinical flare-up of IBD in a number of case reports[1]. Both rectal and colonic frank ulcerations[2], small bowel ulcers[3] and intestinal, diaphragm-like strictures[1,3,4] have been reported after prolonged NSAID intake. On the other hand, no relationship is reported between NSAID treatment and exacerbation of underlying IBD[5,6].

The absence of controlled, prospective trials makes it difficult to draw definitive conclusions. Uncontrolled clinical experience suggests that anti-inflammatory agents can occasionally elicit relapse of IBD[7] and therefore should be employed with caution in patients with either ulcerative colitis or Crohn's disease. A recent systematic review of the available medical literature concluded that the epidemiological evidence for a positive link between NSAID exposure and relapse of IBD is weak, while admitting that "some patients with IBD do relapse when given NSAIDs[8].

Given the inconsistency of the conflicting data concerning the relationship between NSAIDs and IBD, the possible effect of selective cyclooxygenase-2 inhibitors (COXIBs) in this respect remains even more controversial. In order to better understand the relationship between anti-inflammatory treatment and IBD it is necessary to consider the possible pathogenetic mechanisms involved in the adverse effects on the bowel by non-selective NSAIDs. Several mechanisms have been postulated, such as enhanced intestinal permeability[9], enterohepatic recirculation of NSAIDS and formation of drug enterocyte adducts , the latter phenomena having been observed in animal studies[9] but never demonstrated in humans.

The major mechanism involved, however, is thought to be the inhibition of colonic prostaglandin synthesis[10], in particular of the COX-2 isoform. In the inflamed colon COX-2 expression is upregulated in an effort to repair mucosal damage[11] and its inhibition may result in exacerbation of colonic injury and in impairment of the mucosal repair processes elicited by the COX-2 enzyme[12]. In this respect both NSAIDs and COX-2 inhibitors could hamper the progression of the inflammatory state toward healing. On the other hand, if COX-2 is important in the reparative mechanisms in IBD, then patients with quiescent disease should have a lower risk of flare-up when taking NSAIDs[13].

The studies on the effect of COX-2 inhibitors on animal models of colitis have yielded conflicting results[9,14] even taking in account the differences in experimental conditions, type and dosages of the employed compounds. The only available study on human colonic mucosa, carried out on colonic biopsies taken in IBD patients, found that a highly selective COX-2 inhibitor, L-745337 inhibits local release of PGE2 and PGI2 to the same extent as indomethacin, a nonselective NSAID[15], an effect which would likely promote aggravation of mucosal damage..

In a clinical setting a perspective, open-label study in IBD patients with associated arthropathy rofecoxib, administered at a dose of up to 25 mg daily for 20 d, failed to elicit any flare-up of the intestinal disease[16]. Similarly, a retrospective analysis of IBD patients treated with

either celecoxib or rofecoxib for periods ranging from one week to 22 mo[17]. apparently confirmed the safety of COX-2 inhibitors in this respect. By contrast, a clinical exacerbation of the underlying IBD that subsided after the drug was discontinued, has been reported in 19% of patients taking rofecoxib[18]. In keeping with this finding a recent retrospective study in IBD patients taking either celecoxib or rofecoxib has found clinical relapse of the intestinal disease in 39% of cases, again with resolution of symptoms after COX-2 inhibitor withdrawal[19].

On the other hand, the first multicenter, random, double-blind, placebo-controlled study performed in USA ,taking into consideration of both clinical and endoscopic parameters, has shown that celecoxib 200 mg bid for 2 wk is as safe as placebo in patients with ulcerative colitis in remission[20].

Thus, as with nonselective NSAIDs, the available data remain conflicting and confusing. Summing up, on theoretical ground both NSAIDs and COX-2 inhibitors appear capable of triggering a flare-up of IBD by inhibiting the intestinal production of prostaglandins involved in the tissue reparative processes. In clinical practice, although clear-cut evidence is difficult to obtain due to the variable incidence of IBD reactivation and the paucity of prospective, controlled studies, both types of anti-inflammatory agents may precipitate recurrence of intestinal symptoms and therefore should be avoided, when possible, in patients with ulcerative colitis or Crohn's disease.

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REVIEW

Role of radiation therapy in gastric adenocarcinoma

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Abstract

Outcomes in patients with gastric cancer in the United States remain disappointing, with a five-year overall survival rate of approximately 23%. Given high rates of local-regional control following surgery, a strong rationale exists for the use of adjuvant radiation therapy. Randomized trials have shown superior local control with adjuvant radiotherapy and improved overall survival with adjuvant chemoradiation. The benefit of adjuvant chemoradiation in patients who have undergone D2 lymph node dissection by an experienced surgeon is not known, and the benefit of adjuvant radiation therapy in addition to adjuvant chemotherapy continues to be defined.

In unresectable disease, chemoradiation allows long-term survival in a small number of patients and provides effective palliation. Most trials show a benefit to combined modality therapy compared to chemotherapy or radiation therapy alone.

The use of pre-operative, intra-operative, 3D conformal, and intensity modulated radiation therapy in gastric cancer is promising but requires further study.

The current article reviews the role of radiation therapy in the treatment of resectable and unresectable gastric carcinoma, focusing on current recommendations in the United States.

Key words: Radiation therapy; Gastric cancer; Stomach cancer; Chemoradiation; Adjuvant therapy; Neoadjuvant therapy; Intra-operative radiation therapy; 3D conformal radiation therapy; Intensity modulated radiation therapy

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EPIDEMIOLOGY

Worldwide, gastric cancer is the 5th the most common malignancy and the 2nd leading cause of cancer death. Significant geographic variation exists, with high risk areas including Japan, Central and South America, and Eastern Asia, and low risk areas including Kuwait, Israel, and the United States[1, 2]. Despite its relatively low incidence in the United States, gastric cancer is a significant cause of morbidity and mortality, with 22 000 cases per year, resulting in 13 000 deaths[3].

According to the Surveillance, Epidemiology, and End Results (SEER) database, 5-year survival rate for all gastric cancer patients treated in the United States from 1995 to 2001 was 23.2%. This represents an improvement from 15.3% in the time period from 1974-1976[4]. As expected, patients with localized disease have a higher 5-year survival rate (59%) compared to patients with regional (21.9%) or distant disease (3.1%)[4]. Unfortunately, only 25-40% of patients have localized disease at diagnosis.

Outcomes for patients in high risk countries such as Japan are generally better than outcomes in low incidence countries[2, 5]. This variation is likely due to earlier diagnosis (due to aggressive screening programs) and greater clinical experience in high risk countries, as well as differences in the etiology and biology of the tumors in high versus low risk countries[1,2]. The relative contributions of these factors to outcome and the implication for therapeutic decisions remains ill-defined.

Despite the poor prognosis for those patients with gastric cancer, progress has been made in their management. Improved surgical expertise, better systemic therapy, and the integration of radiation therapy have resulted in modest, but significant improvements in local control and survival. The current review article focuses on the role of radiation therapy in the management of gastric carcinoma in the United States.

PATTERNS OF FAILURE

Following surgical resection, both local and distant recurrences are common, indicating the importance of both local and systemic treatment. Patterns of failure have been reported in clinical series, re-operation series, and autopsy series. As expected, recurrences are greatest in autopsy series and least in clinical series, reflecting limitations in

Table 1 Patterns of relapse based on University of Minnesota re-operation series and autopsy series

	Univ. of Minnesota Re-operation series[10] (*n*=107)	Autopsy series[6-9]
Pattern of relapse	%	%
Gastric bed	55	52-68
Anastomosis	27	54-60
Abdominal wound	5	-
Peritoneal seeding	42	30-43
Lymph nodes	43[1]	52
Local-regional as any site	69	80-93
Local-regional only	23	-
Lymph Nodes only	7	-
Lymph nodes only in nodal dissection sites	3	-
Distant metastases	36	50

[1]Most common nodal sites of relapse were porta-hepatis, celiac, and para-aortic regions. Other sites were suprapancreatic, pancreaticoduodenal, and splenic hilar regions.

the ability to detect recurrences clinically. Patterns of recurrence based on re-operation and autopsy studies are summarized in Table 1.

In the University of Minnesota re-operation series gastrectomy patients underwent a "second look" procedure in the absence of known recurrence or a "symptomatic look" in the setting of known recurrence. Local-regional failure alone was detected in 23% of patients and local-regional failure as any component of failure was detected in 69% of patients. Autopsy series report even higher local-regional failure rates (80-93%)[6-9]. These findings suggest that improvements in local-regional control may be potentially curative and provide a strong rationale for adjuvant radiation therapy.

Distant metastases are reported in approximately 50% of patients by autopsy series[6, 7, 9]. The predominant site of distant organ failure is the liver, involved in 30% of patients at initial exploration[5]. Peritoneal seeding, considered distant metastatic disease, is also common, occurring in 42% of patients from the University of Minnesota re-operation series[10], and in 30-43% of patients based on autopsy series[6-9], Lung metastases are less common, observed in only 10% of cases in one autopsy series[5], These findings provide a strong rationale for adjuvant chemotherapy.

ADJUVANT THERAPY

Radiation alone

At least 2 randomized trials compared surgery alone to surgery plus radiation therapy in gastric carcinoma. These results are summarized in Table 2. The first trial, by the British Cancer Stomach Group, randomized patients to surgery alone, surgery followed by radiation therapy, or surgery followed by chemotherapy (mitomycin, adriamycin, and 5-FU). Of note, 40% of patients on this trial had gross or microscopic residual disease following surgery. No survival difference was detected, but the addition of radiation therapy to surgery resulted in a significant reduction in local recurrence (27% with surgery versus 10% with surgery plus radiation therapy)[11, 12]. Interestingly, 24% of patients on the radiation therapy arm did not receive any radiation, and 32% received a dose < 40.5 Gy (well below that required to control microscopic or gross disease). The benefit of adjuvant radiotherapy may be been greater if given to all patients and in adequate doses.

The second trial, by Zhang *et al* in Beijing, randomized 370 patients with gastric cardia carcinoma to neoadjuvant radiation therapy or surgery alone. Neoadjuvant radiotherapy resulted in improved survival (5-year overall survival was 30% *vs*. 20%, $P=0.009$), improved local control (61% *vs*. 48%, $P<0.025$), and improved regional nodal control (61% *vs*. 45%, $P<0.005$)[13]. Additionally, resection rates were higher in the neoadjuvant radiation arm (89.5%) compared to the surgery alone arm (79%, $P<0.01$). Operative mortality and morbidity did not differ between the two arms. While encouraging, this trial included only cardiac lesions, and it is unknown if these results can be generalized to include distal lesions.

While the British and Beijing trials yielded conflicting results regarding a survival advantage with radiation, both demonstrate significant improvement in local control. Local recurrence in gastric cancer has the potential for substantial morbidity, and thus local control alone is a valid endpoint in evaluating the value of neoadjuvant and adjuvant therapy, including radiation.

Chemoradiation

Three randomized trials have compared surgery alone to surgery plus adjuvant chemotherapy and radiation in gastric carcinoma. These trials are summarized in Table 2. The Mayo Clinic trial randomized 62 patients to surgery alone versus surgery plus adjuvant radiation therapy concurrent with 5-FU chemotherapy[14]. A significant improvement in overall survival and disease free survival was identified with the addition of chemoradiation when analyzed by intention to treat. The five-year survival rate for patients randomized to adjuvant chemotherapy and radiation was 23% compared to 4% for those randomized to no adjuvant therapy. When evaluated by actual treatment received, these benefits were no longer statistically significant. Local control was superior in the chemoradiation arm, although this difference did not reach statistical significance.

The trial by Walsh *et al* included primarily patients with adenocarcinoma of the esophagus, but 35% of patients had lesions involving the gastric cardia. Patients were randomized to surgery alone or neoadjuvant chemotherapy and radiotherapy followed by surgery. The addition of chemoradiation resulted in a statistically significant improvement in overall survival[15]. The median survival of patients assigned to preoperative chemoradiation therapy was 16 mo compared with 11 mo for those assigned to surgery alone ($P=0.01$). Three-year survival was 32% versus 6% favoring combined modality therapy ($P=0.01$).

The landmark trial in the combined modality treatment of gastric cancer in the U.S. is Intergroup 0116 study[16]. In this study, 556 patients with resected adenocarcinoma of the stomach or gastroesophageal junction (stage IB through IVM0 disease) were randomly assigned to surgery

Table 2 Randomized trials of adjuvant radiation therapy in gastric carcinoma

Reference	*n*	Survival			Local-regional relapse		
		Median (mo)	5-year (%)	*P* value	%	*P* value	
Mayo Clinic[14]							
Surgery alone		23	15	4	0.05	54	NS
Post-op EBRT + CT		39	24	23		39	
British stomach group[11, 12]							
Surgery alone		145	15	20	NS	27	<0.01 for
Post-op CT		138	13	19		19	
Post-op EBRT		153	17	12		10	EBRT
China-Beijing[13]							
Surgery alone		199	NR	20	0.009	52[1]	<0.025
Pre-op EBRT		171	NR	30		39	
Intergroup 0116[16]							
Surgery alone		275	27	41 (3 yr)	0.005	29	NR
Postop EBRT + CT		281	36	50 (3 yr)		19	
Walsh *et al*[15]							
Surgery alone		55	11	6 (3 yr)	0.01	NR	-
Preop EBRT + CT		58	16	32 (3 yr)			

Abbreviations: CT = chemotherapy, EBRT = external beam radiation therapy, NS = not significant, NR = not reported.

[1]Refers to local relapse. Regional relapse with 54% with surgery alone versus 39% with chemoradiation, $P < 0.05$.

plus postoperative chemoradiotherapy or surgery alone. The adjuvant treatment consisted of two cycles of 5-FU and leucovorin followed by two more cycles of chemotherapy concurrent with radiation therapy. Patients received 45 Gy of radiation in 25 fractions to the preoperative tumor volume, surgical bed, and regional lymph nodes. The majority of patients on this trial had advanced disease (66% were T3/4 and 85% were node positive) and surgical margins were required to be negative.

Three-year overall survival was improved from 41% to 50% with combined modality therapy ($P = 0.005$). The median survival was improved from 27 mo in the surgery only group to 36 mo in the chemoradiotherapy group ($P = 0.005$). Local regional relapse was decreased from 29% to 19% (*P* value not reported). Interestingly, distant metastases were higher in the chemoradiation arm (18% versus 33%), likely a result of patients living long enough to develop clinically detectable distant metastases.

Toxicity was significantly higher with chemoradiation, with nearly three-quarter of patients experiencing grade 3/4 toxicity, and 17% of patients were unable to complete radiation due to toxicity. However, treatment related mortality was low (1% on the chemoradiation arm versus 0% on the surgery alone arm) and overall chemoradiation appeared tolerable in light of its benefits. Postoperative chemoradiotherapy should be considered for all patients at high risk for recurrence of adenocarcinoma of the stomach who have undergone a potentially curative resection.

An important consideration in the interpretation of the Intergroup 0116 trial is the extent of surgery. At least 54% of patients received suboptimal lymph node dissection based on current National Comprehensive Care Network (NCCN) guidelines, raising the question of whether chemoradiation was simply compensating for inadequate surgery. The extent of lymph node dissection as it relates to outcome is discussed further below.

Extent of lymph node dissection and its impact on adjuvant therapy

A D1 lymph node dissection includes perigastric lymph nodes along the lesser and greater curvature. In addition to those nodes included in a D1 lymph node dissection, a D2 dissection includes lymph nodes along the left gastric artery, the common hepatic artery, the celiac trunk, the splenic hilum, and the splenic artery. A D3 lymph node dissection further includes lymph nodes along the hepatoduodenal ligament and the root of mesentery. A D4 lymph node dissection is a D3 dissection plus dissection of para-aortic and para-colic lymph nodes.

While D2 dissection (at a minimum) is considered standard in Japan, randomized Western trials have not shown a survival difference between D1 and D2 dissection, and have demonstrated a substantial increase in post-operative mortality (Table 3)[17, 18]. These trials have been criticized due to the unexpectedly high morbidity and mortality rates, which may be in part explained by the frequent use of pancreatectomy and splenectomy in patients undergoing D2 dissection (which is no longer considered necessary) and by surgeon inexperience[19-21].

In Japan, Maruyama *et al* reported an improvement in 5 year overall survival from 20.3% with D0 dissection to 41.2% with D1 dissection to 63.8% with D2 dissection[22]. Perhaps the greatest criticism of the Japanese data supporting extended lymph node dissection has been its retrospective and/or non-randomized nature. To address these criticisms, the Japanese recently completed a prospective, randomized trial comparing D2 versus D4 dissection. Although disease control data has not yet been reported, acute toxicity data show no difference.[21] Of note, D2 patients had in-hospital mortality rate of 0.8%, which is substantially lower than that reported in the MRC and Dutch trials.

The results of this trial will represent a tremendous contribution to the treatment of gastric cancer. Unfortuna-

Table 3 Randomized trials of D1 versus D2 lymph node dissection in gastric cancer

	MRC[18] (*n*=711)			Dutch[17] (*n*=400)		
Endpoint	D1	D2	*P* value	D1	D2	P value
5 year overall survival	45%	47%	NS	35%	33%	NS
Post-operative morbidity	25%	43%	<0.001	28%	46%	<0.001
Post-operative mortality	4%	10%	0.004	6.5%	13%	0.04

Abbreviations: NS = non-significant.

tely, it is unclear how and if the results of this trial can be applied to Western countries given significant differences in physician experience and patient populations compared to Japan. While surgeon training at tertiary centers in the United States may aid in addressing issues of surgeon experience, hospitals in the United States simply do not have the volume of gastric cancer patients seen in Japan, limiting the ability for surgeons to gain experience. Furthermore, patient related issues such as body mass index and age at diagnosis may differ by geographic location and may influence the risks and benefits of extensive nodal dissection[23].

Evaluation of the benefit of more extended nodal dissection is confounded by stage migration. Bunt *et al* reported that up to 75% of patients with stage IIB disease on D1 dissection are upstaged to IV on D2 dissection[24]. Therefore, when compared stage for stage, results with more extensive nodal dissection may appear superior.

Given these considerations, the optimal extent of lymph node dissection remains elusive. Per NCCN guidelines in the United States, at a minimum a D1 dissection is recommended. Furthermore, it is recommended that at least 15 lymph nodes be evaluated.

On the Intergroup 0116 trial, 10% of patients underwent D2 dissection, 36% underwent D1, and 54% underwent less than a D1 dissection[16]. Subset analysis did not detect differing effects of adjuvant treatment based on the type of lymph node dissection performed (adjuvant treatment appeared to be of benefit regardless of type of lymph node dissection)[25]. This observation is difficult to interpret based on the small number of patients actually treated with D2 dissection. The role of post-operative chemoradiation in patients who undergo D2 dissection by an experienced surgeon is, therefore, unknown.

Lim *et al* reported the outcome of 291 patients treated in Korea with D2 dissection and post-operative chemoradiation identical to that delivered on the Intergroup 0116 trial[26]. 5-year overall survival and local-regional failure were superior to that observed on the chemoradiation arm of the Intergroup 0116 trial, but it is unknown if these outcomes were related primarily to the use of D2 dissection, chemoradiation, or both. It is also unknown if the outcomes were related to surgeon experience and other confounding variables in Korea compared to the United States. The value of chemoradiation in patients who have undergone D2 dissection by an experienced surgeon can only be fully answered by a prospective, randomized trial.

Chemotherapy alone

While the randomized Intergroup 0116 trial showed a benefit in both local control and overall survival with adjuvant chemoradiation, the relative contributions of radiation versus chemotherapy to this benefits are unknown.

The aforementioned British Stomach Group trial, which randomized patients to surgery alone, surgery followed by radiation therapy, or surgery followed by chemotherapy, found no improvement in overall survival with chemotherapy compared to surgery alone or surgery and radiation[11, 12]. A randomized Southwest Oncology Group (SWOG) trial showed no benefit to adjuvant chemotherapy (5-FU, adriamycin, and mitomycin-c) following surgical resection[29]. To date, meta-analyses evaluating the role of adjuvant chemotherapy have demonstrated no or minimal benefit[27, 28].

More recently, however, a British MRC randomized trial (the MAGIC trial) showed an improvement in both overall survival and disease free survival with the addition of pre- and post-operative chemotherapy (epirubicin, cisplatin, and 5-FU) to surgery[30, 31]. Five-year overall survival was improved from 23% to 36% ($P=0.009$). This trial suggests a survival benefit with adjuvant chemotherapy in the absence of radiation therapy. Newer chemotherapeutic regimens may be superior and therefore provide a greater benefit compared to epirubicin, cisplatin, and 5-FU[30, 32-36].

While the results of the MAGIC trial demonstrate a survival advantage to adjuvant chemotherapy in the absence of radiation therapy, only an adequately powered, prospective randomized trial of chemotherapy with or without radiation therapy can fully evaluate the benefit of radiation treatment, and to date no such trial exists. A recent Korean trial treated 81 patients with resection (including D2 dissection) followed by chemotherapy alone (FU, cisplatin) versus chemotherapy (FU, cisplatin) before and after radiation concurrent with capecitabine. With short follow-up (15 mo) there is no difference in disease free or overall survival. Further follow-up is necessary to confirm these results, and radiation therapy should not be omitted on the basis of this trial alone[37].

Currently in the United States, NCCN guidelines recommend adjuvant chemoradiation following complete surgical resection in T3, T4, or node positive gastric cancer patients or in patients with microscopically positive margins. Adjuvant chemotherapy alone or adjuvant radiation therapy alone are considered investigational.

Pre-operative versus post-operative adjuvant radiation therapy

Based on the Intergroup 0116 trial, chemoradiation in the United States is most commonly delivered post-operatively. However, pre-operative radiation therapy offers a number

Table 4 Prospective trials of pre-operative chemoradiation in gastric cancer

Reference	Chemo	RT dose (Gy)	Overall survival % (2 yr)	Median survival time (mo)	Complete response (%)	Partial response (%)	R0 resection (%)	% Distant metastases during therapy	rate (%)
RTOG 99-04[46] (n=49)	5FU/LV/cis×2 prior to RT; 5FU with RT	45	NR	NR	27	NR	77	NR	
Roth[45] (n=19)	Cis/5FU	31.2, 38.4, 45.6[1]	71	NR	5	50	NR	0	
Lowy[41] (n=23)	5FU	45	NR	NR	11	63	NR	17	
Ajani 2004[43] (n=33)	5FU/LV/cis×2 prior to RT; 5FU with RT	45	54	33.7	30	24	NR	12	
Ajani 2005[44] (n=41)	5FU/cis/tax×2 prior to RT; 5FU/tax with RT	45	68[2]	Not reached	20	15[3]	78	0	
Balandraud[47] (n=42)	5FU/cis	45	45.6	23	14	-	81	-	
Klautke[67] (n=21)	Cis/5FU or tax	50.4	42	18	14	62	53	-	

Abbreviations: Cis = cisplatin, 5FU = 5-fluorouracil, LV = leucovorin, tax = paclitaxol, RT = radiation therapy, R0 = resection with negative margins.
[1]Given 1.2 Gy b.i.d. in a phase I dose escalation trial; [2]At a median follow-up of 36 mo; [3]PR defined as <10% residual cells in resected specimen.

of theoretical advantages. First, target tissues are better oxygenated prior to surgery, and radiation therapy is more effective against oxygenated tissues[38]. Second, pre-operative therapy may enhance the ability to perform R0 resection (defined as removal of all tumor such that surgical margins are histologically negative). The MAGIC trial demonstrated an improvement in potentially curative resection rates from 60% with surgery alone to 70% with pre-operative chemotherapy[30], and the Beijing trial demonstrated an improvement in resection rates from 79% with surgery alone to 89.5% with pre-operative radiation therapy[13]. A phase II, multi-institutional trial by the Radiation Therapy Oncology Group (RTOG 99-04) of pre-operative chemoradiation demonstrated a pathologic complete response rate of 27% and an R0 resection rate of 77%, which compares well to historical controls[39, 40]. Table 4 summarizes trials evaluating pre-operative chemoradiation in the setting of gastric carcinoma.

Third, preoperative treatment is generally better tolerated than postoperative treatment and patients are more likely to receive the prescribed doses of chemotherapy and radiation. For example, 88% of patients treated on a phase II trial of pre-operative 5-FU and radiation therapy were able to complete full dose chemoradiation[41], compared to 65% on the Intergroup 0116 trial, which delivered radiation and chemotherapy post-operatively[42]. Furthermore, during pre-operative therapy, distant metastases may manifest clinically and therefore spare patients unwarranted surgery. Prospective trials have reported the development of distant metastatic disease in 12-17% of patients during pre-operative chemoradiation, despite the use of staging laparoscopy at diagnosis[41, 43].

Pre-operative radiation also has potential risks. Perhaps most notably, a significant proportion of patients are under-staged clinically, and based on clinical stage may undergo unnecessary irradiation. Bonenkemp *et al* report that 29% of eligible surgical patients were discovered at laparotomy to have peritoneal, hepatic, or distant lymph node metastases[17]. Careful evaluation including endoscopic ultrasound, CT scans, and laparoscopy is therefore indicated prior to initiation of radiation therapy.

While the potential exists for increased surgical morbidity and mortality with pre-operative treatment, trials of pre-operative chemoradiation have yielded conflicting results in this regard[41, 43-47]. The RTOG 99-04 reported a 21% grade 4 toxicity rate but no treatment-related deaths, demonstrating that pre-operative therapy can be delivered in a multi-institutional setting with acceptable toxicity[46].

The timing of chemoradiation can only be definitively answered through a prospective, randomized trial. An ongoing European trial (SWS-SAKK-43/99) is randomizing gastric cancer patients to pre- versus post-operative chemotherapy; however, this trial does not use radiation and will not address the optimal timing of radiation therapy.

THE ROLE OF RADIATION THERAPY IN UNRESECTABLE GASRTIC CARCINOMA

Only 25-40% of patients with gastric adenocarcinoma will be able to under go potentially curative surgical resection[48]. While the prognosis of these patients is poor, radiation therapy with or without chemotherapy occasionally results in long-term survival (5 year survivals of 5-15%). However, this is not a viable alternative to resection (and adjuvant therapy as indicated) in patients who have resectable disease (Table 5).

Gastric adenocarcinoma is a radioresponsive tumor.

Table 5 Treatment results: Unresectable or residual gastric cancer

Reference	*n*	Treatment	Radiation therapy	Chemotherapy	Results
Randomized					
Mayo Clinic[51]	48	EBRT±CT	35-37.5 Gy in 4-5 wk	5FU 1st wk of EBRT	Median survival 13 *vs* 6 mo and 5-year overall survival 12% *vs* 0% favoring EBRT+5FU
GITSG[53]	90	CT±EBRT	50 Gy split course in 8 wk	5FU during EBRT, maintenance 5FU/MeCCNU	4-year survival 18% *vs* 7%, favoring CT+EBRT
EORTC[52]	90	EBRT±CT	55.5 Gy in 6 wk	5FU	14% long-term survival (3 patients) with EBRT and 5FU
Retrospective					
MGH[55]	32	EBRT±CT	45-55 Gy in 5-6 wk	5FU during EBRT, maintenance 5FU/MeCCNU	EBRT+CT: Unresectable disease 14 mo median survival; unresectable and residual disease 10% 4-year survival

Abbreviations: EBRT=external beam radiation therapy; CT=chemotherapy; 5FU=5-fluorouracil; MeCCNU=semustine; GITSG=gastrointestinal study group; EORTC=European organization for research and treatment of cancer; MGH=Massachusetts General Hospital; Gy=gray.

Wieland and Hymmen used radiotherapy alone in patients with unresectable gastric cancer. The radiation dose was 60 Gy when feasible, in 1.5 to 2.0 Gy fractions. They noted an 11% (9 of 82) 3-year and 7% (5 of 72) 5-year survival[49]. Abe and Takahashi reported a 14.7% 5-year survival rate with intraoperative radiation therapy (28 to 35 Gy) in a group of 27 patients with stage IV disease. In the same study, there were no 5-year survivors in the 18 stage IV patients randomized to a surgery-alone control arm[50].

Most phase III trials for unresectable or residual gastric cancer show an advantage for combined-modality treatment over single-modality treatment. Two randomized trials compared radiation therapy alone to chemoradiation. The seminal trial in the treatment of unresectable gastric cancer is from the Mayo clinic[51]. All patients were randomized to radiotherapy (35 to 37.5 Gy in 4 to 5 wk) with or without 5-FU chemotherapy during the first 3 d of radiation. Mean and overall survival was improved in the combined-modality treatment group compared to radiotherapy alone (13 *vs* 6 mo median survival and 12% *vs* 0% 5-year survival, respectively). The EORTC performed a randomized trial of radiation therapy with or without 5-FU chemotherapy following attempted surgical resection[52]. Residual disease after resection was identified in 22 patients and the three long-term survivors (14%) received both irradiation and 5-FU.

Two randomized trials compared chemotherapy alone to chemoradiation. In a study by the GITSG, 90 patients were randomized to radiation therapy and 5-FU followed by maintenance 5-FU/MeCCNU or 5-FU/MeCCNU alone[53]. The radiotherapy dose was 50 Gy given in a split course. This trial included patients with unresectable, gross, microscopic, or no residual disease after resection, though only 7 patients had no residual disease. Radiation and chemotherapy resulted in a statistically significant improvement in 4-year survival compared with chemotherapy alone (18% *vs* 6%, respectively). A second trial performed by the GITSG comparing radiation and chemotherapy to chemotherapy alone did not demonstrate a survival advantage to combined modality therapy[54]. However, the results from this second trial are difficult to interpret as nearly 50% of patients on the combined modality arm either did not receive the prescribed dose of radiation or had a major deviation in radiation delivery.

Data from retrospective series also suggest an improvement in disease control and survival with the combination of radiation therapy and chemotherapy. In published series from the Mayo Clinic and Massachusetts General Hospital (MGH), long-term survival of 10% or more has been demonstrated in patients receiving external beam radiation and chemotherapy following subtotal surgical resection with residual disease (MGH) or unresectable disease[55,56]. In the MGH experience, patients with unresectable or gross residual disease treated with combined radiation and chemotherapy had median survivals of 14 mo and 15 mo, respectively, and the overall four-year survival rate was 10%.

In the University of Pennsylvania experience with unresected adenocarcinoma of the GE junction or esophagus, both local control and overall survival was superior with combined modality versus single modality therapy[57]. Local control was 52% with radiation and chemotherapy, compared to 4% with radiation alone and 0% with chemotherapy alone. Median survival with radiation and chemotherapy was 10 mo compared with 5 mo for radiation alone. Overall, considering the randomized and retrospective data, there appears to be improved survival with combined radiation and chemotherapy for unresectable gastric cancer. However, the use combined-modality therapy in unresectable gastric cancer is tempered by the fact that relatively few of these patients will be long-term survivors.

To the best of our knowledge, there are no randomized trials of neoadjuvant radiation and chemotherapy in unresectable gastric adenocarcinoma. Neoadjuvant chemotherapy alone has been used in unresectable and locally advanced disease in numerous phase II and in at least one phase III trial. Wilke *et al* treated 34 patients with unresectable gastric cancer confirmed at exploratory laparotomy with etoposide, adriamycin and cisplatin chemotherapy[58]. Following chemotherapy and second look operation, fifteen patients (44%) underwent potentially curative resection and five patients (15%) were pathologic complete respon-

ders. Median survival in this phase II trial was 18 mo for all patients. In an update, 79% of these patients had local-regional failure[58].

Taken as a whole, neoadjuvant chemotherapy for unresectable gastric cancer results in clinical response rates of 30% to 68%, potentially curative resections in as few as 8% or as many as 73% of patients, high rates of local relapse, and except for the Wilke study, pathologic complete response rates of <15%. In view of the high incidence of local recurrence and relatively low pathologic complete response rates, radiation therapy has been integrated into neoadjuvant studies of unresectable gastric cancer. A recent US Intergroup trial, to confirm the Walsh trial[15], of neoadjuvant combined-modality therapy in esophageal and GE junction carcinoma was closed due to inadequate accrual.

In the purely palliative setting for gastric cancer, total or subtotal gastric resection can successfully relieve symptoms of obstruction, hemorrhage, and ulceration. In advanced gastric cancer, total gastrectomy for bulky or proximal tumors has resulted in good quality of life, but was less helpful for relief of symptoms due to linitis plastica[59]. In the absence of surgery, radiation therapy is effective in 50% to 75% of patients in the palliation of gastric outlet or biliary obstruction, pain, and bleeding[48]. In select patients with good performance status, the administration of concurrent 5-FU chemotherapy may improve response.

RADIATION THERAPY TECHNIQUES

Intra-operative radiation therapy

Intraoperative radiation therapy (IORT) delivers a single high dose of radiation to areas at high risk relapse. A substantial advantage of intraoperative radiation therapy is that normal tissues can be largely excluded from the radiation field, such that larger doses of radiation can be delivered to the target tissues and toxicity minimized. Although appealing, the role of intraoperative radiation therapy in the setting of gastric cancer remains ill-defined. Several studies suggest an advantage to IORT in locally advanced gastric carcinoma, but these trials do not define the role of IORT in patients receiving EBRT.

A randomized trial by Sindelar *et al* compared gastrectomy with IORT to gastrectomy with or without EBRT (EBRT was not used in early stage lesions confined to the stomach) in forty-one patients[60]. Survival was equivalent between the 2 arms, but local-regional control was superior on the IORT arm versus the control arm (64% versus 8% respectively; $P < 0.001$). Skoropad *et al* randomized patients with gastric cancer to pre-operative EBRT (20 Gy in 5 d) followed by IORT (20 Gy) plus gastrectomy versus gastrectomy alone[61]. The addition of radiation therapy had a statistically significant survival advantage in more advanced stages (T3-4 and/or node positive). In a randomized trial of 211 patients from Kyoto, Japan, patients were randomized to 28-35 Gy of IORT or surgery alone[62]. This study also demonstrated improved survival in patients with disease penetrating the wall of the stomach or in patients with involved lymph nodes, but not in patients with disease limited to the wall of the stomach. While these trials suggest an advantage to radiation therapy in locally advanced disease, they do not answer the question of whether EBRT alone, IORT alone, or EBRT plus IORT should be used. To date, no randomized trials exist evaluating EBRT with or without IORT.

Notably, prospective and retrospective trials have not demonstrated increases in morbidity and mortality with IORT[63]. Given its potential benefits and limited morbidity, IORT in the setting of gastric cancer continues to be used in select centers in the United States.

3D conformal and intensity modulated radiation therapy (IMRT)

Radiation therapy technique is important in evaluating the benefits of radiation. In the Intergroup 0116 trial, 2D radiation therapy with an anterior-posterior (AP) and a posterior-anterior (PA) radiation beam was utilized. Radiation therapy portals were centrally reviewed prior to radiation, and 34% had major protocol violations prior to revision. These findings demonstrate that trials without central review may suffer from inadequate radiation therapy field design, and suggest that the skill and experience of the treating radiation oncologist is important.

Technology has evolved over time to allow increasingly conformal radiation therapy, potentially sparing normal tissues and decreasing toxicity and/or allowing dose escalation to the target volume. However, as technology allows progressively more conformal treatment, the radiation oncologist must be increasingly mindful of known patterns of recurrence.

Two such targeted techniques are 3D conformal radiation therapy and intensity modulated radiation therapy (IMRT). 3D conformal radiation therapy uses 3D reconstruction of the target volume and normal tissues from CT data to plan multiple radiotherapy beams conforming to the target and sparing, as much as possible, normal tissues. Leong *et al* compared a 6-field 3D conformal radiation therapy plan a 2D AP:PA radiation therapy plan in 15 patients and noted lower radiation doses to the kidneys and spinal cord, both important radiation dose-limiting normal tissues[64]. Dose was higher to the liver with the 3D plans, but still well below tolerance. Princess Margaret Hospital treated 50 patients per the chemoradiation arm of the INT 0116 protocol, but used a 5-field 3D conformal radiation technique. Grade 3 or 4 toxicity occurred in 52% of patients, similar to the INT 0116 trial, suggesting minimal impact on toxicity compared to 2D radiation therapy.

Intensity modulated therapy, like 3D conformal radiation therapy, uses multiple beams with the shape of each beam conforming to the target. Unlike 3D radiation therapy, the intensity of radiation within any given beam varies such that normal organs are spared compared to target tissues. Planning studies of IMRT in the use of gastric cancer demonstrate decreased dose to the kidneys compared to conventional plans[65-67]. In treating abdominal organs, however, IMRT must be used with caution as intra-abdominal motion can be significant and highly conformal treatments can miss targeted areas. The role of IMRT in gastric cancer is not yet defined and remains investigational.

To date, 3D conformal and IMRT techniques have not

demonstrated a convincing reduction in acute or late radiation toxicity. However, both techniques offer great potential to decrease radiation toxicity by avoiding normal tissues and to allow dose escalation to target tissues.

CONCLUSION

From the available clinical data in the treatment of gastric carcinoma, the following conclusions can be reached: (1) Patterns of failure following surgical resection demonstrate high local and distant failure rates, suggesting a need for both local and systemic adjuvant treatment. (2) Adjuvant radiation therapy improves local control based on randomized trials. (3) Adjuvant chemoradiation improves survival in the randomized Intergroup 0116 trial, and adjuvant chemoradiation following complete resection with negative margins is currently considered standard of care in the United States. (4) Chemoradiation allows long-term survival in a small number of patients with locally advanced, unresectable gastric carcinoma.

However, each advancement in the treatment of gastric cancer leads to further questions, which should serve as the basis for future clinical trials: (1) In the adjuvant or neoadjuvant setting, what is the benefit of adding radiation therapy to chemotherapy? (2) Does adjuvant chemotherapy and/or radiation therapy provide benefit in patients who have undergone a D2 lymph node dissection? (3) Is pre-operative radiation therapy superior to post-operative radiation therapy? (4) Will conformal radiation technologies including IORT, 3D conformal, and intensity modulated radiation therapy improve outcomes?

Despite its disappointing survival rates, the treatment of gastric carcinoma has improved modestly over time. Radiation therapy has a role in the adjuvant setting for improving local control and, in combination with chemotherapy, survival. Radiation therapy in unresectable disease provides effective palliation and, in combination with chemotherapy, long term survival in a small number of patients.

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REVIEW

Pathogenesis of columnar-lined esophagus

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Abstract

Since its initial description, the pathogenesis of the columnar-lined esophagus (CLE) has been surrounded by many controversies. The first controversy is related to the existence of the condition itself. The second controversy centers on whether the CLE is a congenital or an acquired condition. In this article, we review the congenital and acquired theories of development of CLE and discuss the various factors in acquisition of CLE. The bulk of evidence in the literature suggests that CLE is an acquired condition.

Key words: Barrett's esophagus; Risk factors; Esophageal adenocarcinoma; Pathogenesis; Reflux esophagitis; Gastroesophageal reflux

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INTRODUCTION

The lower esophagus lined by columnar epithelium is a condition poor in fact but rich in theory. It invites speculation but resists laboratory reproduction[1]. Columnar-lined esophagus (CLE) is commonly called "Barrett's esophagus". The same eponym is also used to describe the metaplastic epithelium and complications of this condition. Barrett's epithelium, Barrett's mucosa, Barrett's ulcer, Barrett's stricture and Barrett's carcinoma are used frequently in the literature. It is a pathological premalignant condition in which the lower segment of the esophagus becomes lined by a metaplastic columnar epithelium in the form of either circumferential extension of the gastric columnar epithelium or islands of columnar mucosa or both. The presence of specialized columnar epithelium anywhere in the esophagus represents a true metaplasia of the normal squamous epithelium and is highly suggestive for the diagnosis of CLE.

Since its initial description, the pathogenesis of CLE has been surrounded by many controversies. The first controversy is related to the existence of the condition itself. For more than six years, Norman Barrett[2,3] argued that the columnar-lined structure is an intrathoracic stomach due to a congenitally short esophagus. Earlier in 1943, Allison *et al*[4] described peptic ulceration in the 'short' esophagus. Allison[5] introduced the term reflux esophagitis and showed that peptic ulcer is in the esophagus. Our current concept of CLE is based on the demonstration by both Allison and Johnstone[6]. They have proven that the columnar-lined structure is esophagus on the basis of the following criteria: (1) The tubular structure grossly resembles the esophagus; (2) absence of peritoneal covering; (3) presence of squamous islands within the columnar mucosa; (4) presence of muscularis propria and submucosal glands of esophageal type; and (5) absence of the acid-producing oxyntic cells in the gastric mucous membrane, which line the lower esophagus.

The second controversy center on whether the CLE is a congenital or an acquired condition.

CONGENITAL THEORY OF COLUMNAR-LINED ESOPHAGUS

This theory is based upon the fact that the embryonic esophagus has a columnar epithelial lining, which may later be incompletely replaced with squamous epithelium leaving the lower esophagus lined by the fetal columnar epithelium. Many investigators showed that the embryonic columnar and glandular epithelium might persist in the esophagus after birth. Islets of ciliated columnar epithelium have been reported in post-mortem examination of esophagus from premature and newborn infants. There are four types of congenital columnar epithelium in the esophagus.

Embryonic ciliated columnar epithelium

Johns[7] showed that the fetal esophagus is lined by ciliated columnar epithelium until the 17th wk of development, and is later replaced by squamous epithelium. Initially, this replacement starts in the middle of the esophagus and then extends in both directions. Although, at birth, the esophagus is entirely lined by squamous epithelium, remnants of this embryonic ciliated columnar epithelium sometimes remain in the esophagus after birth. Rector and Connerley[8] found such columnar epithelium in the

esophagus of 7.8% of infants and children. It was more than 8 times commoner in the upper end than the lower end. A sheet of ciliated columnar epithelium has also been found lining the lower esophagus of one asymptomatic adult[9].

Heterotopic or ectopic gastric epithelium

Occasional foci of gastric epithelium were also reported to be present in the esophagus. Carrie[10] reported islands of ectopic gastric mucosa in 50% of his patients. Bosher and Taylor[11] reported the presence of heterotopic gastric mucosa associated with esophageal ulcer and stricture formation. Moreover, Abrams and Heath[12] also showed such epithelium.

Superficial cardiac glands

Normally superficial cardiac glands are present in the lamina propria, particularly in the lower end of the esophagus.

Congenital Barrett's epithelium

Although there is no similarity between the ciliated columnar epithelium and Barrett's epithelium, some investigators considered Barrett's epithelium to be a congenital remnant of the ciliated columnar epithelium.

Initially, Norman Barrett argued that since the early embryonic esophagus is lined by columnar epithelium, and that the metamorphosis to squamous epithelium only occurs after proliferation and recanalization of the original layer, the presence of columnar epithelium in the esophagus of an adult might be explained on the basis of a failure of this process to achieve completion[13]. Later on in 1962, Barrett changed his views and argued that the difficulty in accepting this explanation is that if it were true, one might expect the whole esophagus, and not simply the lower end to be abnormally lined more often than it is[14].

EVIDENCE SUPPORTING CONGENITAL ORIGIN OF COLUMNAR MUCOSA

Epidemiological evidence

Several studies have reported the cases of CLE in patients who had no GER[21-23]. Many authors reported the cases of CLE in children[24-26]. Two reports showed that a 9-year-old boy[24] and a 10-year old girl[24] had experienced dysphagia since infancy. The authors who favored the congenital theory assumed that the columnar mucosa is present since birth, but it becomes symptomatic later in life as a result of the development of a hiatal hernia and GER. Only a very small proportion of patients with CLE develop esophageal adenocarcinoma.

Morphological evidence

Postlethwait and Musser[27] in an autopsy study described a neonate with CLE. Haque and Merkel[28] reported a case of total CLE, where an adult patient had typical Barrett' s mucosa involving the esophagus. The authors argued that such a case support a congenital origin. However, a review of this case shows that this patient had long-standing, severe GER requiring antireflux surgery. Wolfe *et al*[29] and Mangla[22] described the presence of islands of heterotopic/ectopic gastric mucosa in the esophagus. Mangla[22] also described the cases with squamous epithelium interposed between Barrett's and gastric mucosa. Stadelmann *et al*[23] described patients with a linear, smooth squamo-columnar junction rather than the irregular junction commonly found in patients with CLE, and they argued that this observation support the congenital origin. Other several studies[22,30,31] argued that the presence of parietal and chief cells in Barrett's mucosa, which are highly differentiated cells, is unlikely to be an acquired condition.

Experimental evidence

Failure of experimental models has been reported by Van de Kerckhof and Gahagan by injuring the esophageal mucosa[32]. The absence of reversion of Barrett's mucosa to squamous epithelium after antireflux surgery has been cited as evidence supporting a congenital origin[22].

Details of evidence for production of CLE experimentally will be presented when discussing the acquired origin of columnar mucosa.

ACQUIRED THEORY OF COLUMNAR-LINED ESOPHAGUS

The arguments against the congenital theory include the fact that the embryonic studies have shown that the squamous replacement of the fetal columnar epithelium begins in the mid esophagus and progresses toward each end[7]. In addition, despite the fact that the islets of congenital ectopic gastric mucosa are more common in the cervical region of the esophagus which appears to be the last area to lose the embryonic columnar lining, the columnar epithelium is always found in the lower esophagus.

The association between CLE and gastroesophageal reflux (GER) was recognized early and led to the concept that CLE is an acquired condition. Tileston[15] suggested that the first requisite for the formation of peptic ulcer of the esophagus is an insufficiency of the cardia. He concluded that hyperacidity of the gastric juice plays a part in preventing healing of the esophagus ulcer seems probable. In the same article, he noticed with interest the close resemblance of the mucous membrane around the esophageal ulcer to that normally found in the stomach. In their historical paper, Allison and Johnstone[6] concluded that peptic esophagitis and peptic ulceration of the squamous epithelium of the esophagus are secondary to regurgitation of digestive juices, are most commonly found in those patients in whom the competence of the cardia has been lost through herniation of the stomach into the mediastinum. In the past, there has been some discussion about gastric heterotopia as a cause of peptic ulcer of the esophagus, but this point was very largely settled when the term reflux esophagitis was coined. It describes accurately in two words the pathology and etiology of a condition which are a common cause of digestive disorder. They reported seven patients in whom the lower esophagus

Table 1 Various evidences to support the congenital and acquired theories

Evidences	Congenital theory	Acquired theory
Epidemiological	1 Cases in children	1 Association with gastroesophageal reflux
	2 Cases without demonstrated reflux	2 Usual occurrence after middle age
Morphological	1 Neonate with columnar-lined esophagus	1 Absence of typical cases in fetuses
	2 Adult with involvement of entire esophagus	2 Presence of intestinal-type goblet cells and sulphomucins.
	3 Islands of heterotopic/ectopic gastric mucosa	3 Absence of gastrin-containing cells.
	4 Cases with squamous epithelium interposed between Barrett's and gastric mucosa	4 Endoscopic demonstration of upwards migration of Barrett's mucosa with ongoing gastroesophageal reflux
	5 Cases with linear, smooth squamo-columnar junction	
	6 Presence of parietal and chief cells in Barrett's mucosa	
Experimental	1 Failure of experimental models	1 Animal models.
	2 Absence of reversion to squamous epithelium after antireflux operation	2 Regression after successful antireflux surgery.
		3 Acquisition of Barrett's mucosa after onset of reflux following esophagogastrostomy, Heller myotomy and esophagojejunostomy.

Modified from Hamilton SR. Pathogenesis of columnar cell-lined (Barrett's) esophagus. In: Spechler S J, Goyal R K, (Eds.) Barrett's esophagus: Pathophysiology, diagnosis, and management. New York: Elsevier Science, 1985: 29-37.

was lined by gastric mucous membrane, and all these patients had reflux esophagitis. Four of these patients had progressed to stricture formations and one patient had ulceration in the esophageal part, which was lined by gastric epithelium. They termed such ulcers as Barrett's ulcer although they stressed that the use of this eponym does not imply agreement with Barrett's description of an esophagus lined with gastric mucous membrane as "stomach".

Although Allison and later on Barrett recognized the association between CLE and hiatal hernia and reflux esophagitis, nevertheless, they assumed that the condition is congenital in origin. Moersch *et al*[16] were the first to suggest that the columnar lining might be an acquired condition as a sequel of reflux esophagitis. Hayward[17] suggested that the CLE is an acquired condition due to reflux esophagitis that destroys the squamous epithelium. Moreover, he suggested that the denuded area is re-epithelialized from the columnar cells below, and that the lower 1 to 2 cm of the esophagus is lined by columnar epithelium which acts as a buffer zone of junctional epithelium, does not secrete acid or pepsin but is resistant to them.

The principal of the acquired theory is that the columnar epithelium is an adaptive change in response to the continued harmful environment present in the esophagus due to ongoing GER. Following repeated cycles of destruction and regeneration, in some individuals, a simple columnar epithelium more resistant to digestive action finally replaces the destroyed squamous epithelium[1].

Many earlier observations documented the ascent up the esophagus of a stricture at the squamo-columnar junction as squamous epithelium is replaced by columnar epithelium, supporting the concept of an acquired dynamic epithelium[18,19]. Adler[1] reported a series of 11 patients with CLE associated with strictures, in three of them, progression and ascent of the strictures were documented over 3 to 5 years. Moreover, he also suggested that the occurrence of islets of squamous epithelium within the sheet of columnar epithelium could be interpreted as the more resistant remnants of squamous epithelium that survived the erosive action of the digestive secretions. He stated that the findings of these squamous islets are less easily explained by the congenital theory.

Hamilton[20] summarized the epidemiological, morphological and experimental evidence cited in the literature to support the congenital and acquired theories (Table 1).

EVIDENCE SUPPORTING ACQUIRED ORIGIN OF COLUMNAR MUCOSA

Epidemiological evidence

(1) Many investigators reported the frequent association between CLE and GER[33]. Allison and Johnstone[6] suggested that if gastric reflux occurs, and causes ulceration of squamous epithelium without producing stenosis, is healing of the ulcer in an acid medium more likely to be by overgrowth of gastric rather than of esophageal epithelium? If this were so it might be that some examples of the gastric mucosa in the esophagus were acquired rather than congenital. (2) The usual occurrence of CLE in patients of middle and older ages. If the congenital columnar epithelium lining the esophagus produces acid and pepsin itself as some believe it is difficult to understand why complications do not occur early in life. (3) Dahms and Rothstein[34] reported that CLE in children is a consequence of chronic GER.

Morphological evidence

There is no typical cases of CLE in fetuses[33]. The histopathologic and histochemical characteristics of Barrett' s epithelium have also been quoted to support an acquired origin. The presence of intestinal-type goblet cells, and sulphomucins has suggested an acquired origin[35,36]. Dalton *et al*[37] and Dayal and Wolf[38] suggested that the absence of gastrin-containing cells from Barrett's mucosa as contrasted with congenital gastric heterotopia such as in a Meckel's diverticulum, support an acquired origin for CLE. However, Mangla *et al*[30] have demonstrated the presence of gastrin in Barrett's mucosa. The strongest morphological evidence for acquired origin has been

provided by cases with endoscopic demonstration of cephalic migration of the peptic esophagitis and stricture above an ascending boundary of CLE with continuing GER[19,26,39]. These cases provided strong evidence that the condition could be progressive and result from persistent severe GER and complicating esophagitis. The concept of erosive reflux esophagitis healing by upward migration of adjacent columnar epithelium was advanced by Hayward[17]. Adler[1] reported that out of five patients with CLE associated with strictures, three patients had progression and ascent of the strictures, which were documented over 3 to 5 years. He proposed the possibility of repair by extension from esophageal glands following reflux esophagitis.

Experimental evidence

Bremner *et al*[40] and Wong and Finckh[41] successfully produced columnar-lined mucosa in the esophagi of dogs and rats in the presence of GER, respectively. Li *et al*[42] showed that seven of the 10 dogs developed Barrett's mucosa after a reflux procedure was created, suggesting that a squamous injury in the esophagus is repaired by columnar epithelium in the presence of reflux. The columnar epithelium is acquired when the squamous epithelium is injured and during repair it undergoes columnar metaplasia. Although damage to squamous mucosa is a requisite factor for the development of columnar metaplasia, acute injury alone is not a sufficient stimulus for columnar metaplasia. The acquisition of the columnar epithelium requires a chronically abnormal esophageal environment during the period of mucosal repair. In an experimental study on dog esophagus, Gillen *et al*[43] found that a surgical injury in the esophageal squamous mucosa healed by the regeneration of squamous epithelium in normal dogs. However, if the surgical injury is preceded by alteration of the gastroesophageal junction to cause chronic GER, then the injured squamous mucosa is replaced by a columnar lining. Radigan *et al*[44] and Ransom *et al*[45] reported restoration of squamous mucosa after a successful antireflux operation. The acquisition of Barrett's mucosa after onset of GER following esophagogastrostomy, Heller myotomy, and esophagojejunostomy provide strong evidence for an acquired origin[46-49].

The arguments regarding the etiology of CLE have centered on an acquired *versus* congenital origin; the possibility remains that examples of both do occur[50]. However, the available evidence suggests that the vast majority of cases, if not all, are acquired due to chronic GER. The question remaining to be answered is why Barrett's mucosa develops in only 8-20% of patients with reflux, but not in other patients with reflux of similar severity[20].

FACTORS IN ACQUISITION OF CLE

Patients with CLE frequently have anatomical and physiological abnormalities which either predispose to or exaggerate the severe GER, which in turn leads to the acquisition of the columnar epithelium. Another group of these patients acquire the columnar epithelium secondary to esophagitis or mucositis due to irritant or cytotoxic agents. Given the combination of these anatomical and physiological abnormalities, it is not surprising that severe esophagitis commonly accompanies Barrett's epithelium.

The major risk factors that can lead to acquisition of the columnar epithelium are: (1) GER with acid and pepsin; (2) extreme lower esophageal sphincter incompetence; (3) alkaline reflux (duodeno-gastroesophageal reflux of bile and pancreatic juices); (4) gastric acid hypersecretion; (5) abnormal esophageal motility and delayed acid clearance; (6) diminished esophageal pain sensitivity; and (7) alcohol consumption.

The minor risk factors that can lead to acquisition of the columnar epithelium are: (1) Caustic injury by lye; (2) cytotoxic chemotherapy agent; and (3) acid and pepsin secretion by Barrett's epithelium.

GER with acid and pepsin

Much attention has been directed to reflux of the gastric juices containing acid and pepsin. Patients with CLE frequently have an extremely incompetent lower esophageal sphincter, and therefore, are exceptionally predisposed to GER. In most patients with CLE, GER is judged to be the principal factor that causes injury to the squamous epithelium and provides the abnormal esophageal environment necessary for columnar metaplasia. It is now widely accepted that a substantial proportion of patients with Barrett's esophagus do have severe GER. Schnell *et al*[51] conducted a study to test the hypothesis that since acid contact time is related to formation of Barrett' s epithelium, then a greater esophageal acid contact time might result in longer segments of Barrett's epithelium. They studied 116 patients with CLE and demonstrated a definite positive correlation between the length of Barrett' s esophagus and the duration of esophageal acid exposure. Recently some of the published Swedish studies linked adenocarcinoma of esophagus with duration, frequency and severity of GER symptoms[52,53].

Extreme lower esophageal sphincter incompetence

An important factor that contributes to mucosal injury in patients with CLE is lower esophageal sphincter (LES) hypotension. Stein *et al*[54] found that 11 of 12 patients with CLE had an incompetent LES. The abnormalities were defined as LES pressure of less than 6 mm Hg, overall LES length of less than 2 cm, or abdominal LES length of less than 1 cm. They concluded that this predisposes to GER. This study also confirmed earlier reports documenting extreme LES hypotension in patients with CLE. Attwood *et al*[55] reported that 90% of patients with CLE had a mechanically defective LES and 93% had increased esophageal exposure to gastric juice on esophageal pH monitoring.

Alkaline reflux (duodeno-gastro-esophageal reflux of bile and pancreatic juices)

Most of the earlier studies on CLE traditionally focused on the role of gastric acid and pepsin, but other reports suggested that other factors contributed to the esophageal damage in patients with CLE. Bile reflux into the esophagus was reported as an important causative

factor in acquisition of CLE by many investigators[39,47]. DeMeester *et al*[56] using 24-h esophageal pH monitoring found abnormal alkaline reflux (esophageal pH >7 for more than 10.5% of a 24-h period) in 34% (14/41) cases. Gastric secretions normally are not alkaline, suggesting that bile and other alkaline materials in the duodenum frequently reflux into the stomach and esophagus in patients with CLE and this alkaline reflux has a role in the esophageal injury. Waring *et al*[57] also documented the frequent occurrence of duodeno-gastric reflux in patients with CLE. In their study, radionuclide appeared within 95 min of an intravenous injection of ^{99m}Tc-diisopropyl iminodiacetic acid in the stomach of 46% (6/13) patients with CLE. In contrast, 11% (2/19) persons in the control group had radionuclide evidence of duodeno-gastric reflux. The role of alkaline reflux is particularly evident in those cases of CLE acquired after total gastrectomy in which gastric juices were eliminated[49]. Tada *et al*[58] also provided evidence that refluxed substances other than acid and pepsin play a pathogenic role in CLE. They reported a patient with severe GER disease who developed CLE at age 47, 22 years after a total gastrectomy. At age 64, an endoscopic examination revealed adenocarcinoma in Barrett's epithelium. This report confirms previous reports that acid and pepsin are not necessarily the only pathogenic factors for either Barrett's esophagus or esophageal adenocarcinoma, and further supports the important role of alkaline reflux in the development of CLE and adenocarcinoma. Stoker and Williams[59] suggested that a mixture of gastric and duodenal secretions causes esophageal mucosal disruption and intracellular damage through a toxic synergism between the two secretions. The clinical importance of the alkaline reflux is that the treatment of GER in patients with CLE should involve shifting or neutralizing the alkaline substances as well as the acid. As with duodeno-gastric reflux, the stomach contents available for reflux into the esophagus will include bile and pancreatic enzymes in addition to acid. Therefore, suppressing the acids alone may be ineffective in healing esophagitis for patients with CLE. Hetzel *et al*[60] showed that omeprazole, a drug that is dramatically effective in reducing gastric acid secretion and in healing mild esophagitis, was far less reliable for healing ulcerations in Barrett's epithelium. van der Veen *et al*[61] reported that previous gastric surgery represents an increased risk factor for the development of adenocarcinoma in patients with CLE. Attwood *et al*[62] conducted a study to determine the role of intra-esophageal alkalinization in the genesis of complications in Barrett's esophagus. They found that the esophageal acid exposure (% time pH<4) was similar in patients with or without complications (19.2% *vs* 19.3%). In contrast, the esophageal alkaline exposure (% time pH>7) was greater in patients with complications (24.2% *vs* 8.4%). In 81% of patients, there was a clear relationship between gastric and esophageal alkalinization. They concluded that complications in Barrett's esophagus develop in association with increased exposure of the esophagus to an alkaline environment, which appears to be secondary to duodeno-gastric reflux.

Gastric acid hypersecretion

Patients with CLE may have elevated gastric acid secretion. Collen *et al*[63] suggested that hypersecretion of gastric acid may contribute to the frequency of complications in Barrett's esophagus. In a prospective study, 75% (9/12) patients who had heartburn intractable to treatment with conventional doses of ranitidine (150 mg twice per day) were found to have gastric acid hypersecretion (basal acid output > 10 mEq/h). Among those 12 refractory patients, 10 had Barrett's esophagus, and increasing the conventional dose of ranitidine 2 to 6 times controlled their heartburn, which reduced the gastric acid output to less than 1 mEq/h. With gastric acid hypersecretion and duodeno-gastric reflux of bile and pancreatic enzymes, the gastric material available for reflux can be unusually caustic and harmful.

Abnormal esophageal motility and delayed acid clearance

Many patients with CLE have abnormal motility in the distal esophagus that can delay esophageal clearance, and so the refluxed material may have prolonged contact with the esophageal mucosa. Winter *et al*[64] reported on the importance of delayed acid clearance from the esophagus as a contributory factor in the causation of CLE. Stein *et al*[54] using 24-h esophageal motility monitoring reported that there was an increase in the frequency of abnormally weak contractions (<30 mm Hg) of the distal esophagus in patients with CLE compared with patients with uncomplicated reflux disease. These weak contractions may not empty the esophagus effectively, and could be responsible for the delayed esophageal acid clearance that has been observed in patients with Barrett's esophagus.

Diminished esophageal pain sensitivity

There are large proportions of patients with CLE who are asymptomatic. Cameron *et al*[65] estimated that for every case identified clinically, there are approximately 20 cases in the general population that go unrecognized. Patients with CLE usually seek medical advice when they develop complications, such as esophagitis, stricture, ulcer, bleeding or adenocarcinoma. Many patients with CLE have diminished esophageal pain sensitivity. Skinner *et al*[66] suggested that persistence of severe reflux after antireflux surgery in some patients with Barrett's esophagus who are asymptomatic is due to the Barrett's epithelium not being sensitive to acid after ulceration or strictures heal. Iascone *et al*[67] found that as compared to patients with reflux esophagitis without Barrett's epithelium, patients with Barrett's esophagus have less severe symptoms of heartburn and regurgitation in spite of significantly greater duration of acid reflux in the distal esophagus. Johnson *et al*[68] suggested that the CLE has diminished pain sensitivity and that acid reflux may not cause heartburn for many patients with CLE. Perhaps most patients with Barrett's esophagus escape medical attention because their reflux symptoms are minimal. Diminished pain sensitivity may play a role in the development of the complications that frequently accompany CLE. Patients whose reflux is not heralded by heartburn are unlikely to take antacid or seek medical advice. With no symptomatic warning that caustic material is damaging the esophagus, patients are unlikely to take medications that may prevent further injury[69]. The decrease in pain sensitivity may explain the

lack of reflux symptoms before the onset of dysphagia in some of the patients who develop adenocarcinoma.

Alcohol consumption

Martini and Wienbeck[70] noted the frequent history of chronic alcohol abuse in patients with CLE. Whether this association is related to a direct effect of alcohol on the mucosa or to promotion of GER by the effects of alcohol on the LES is uncertain. It is interesting to notice that alcohol abuse was quoted as early as 1906 as an important cause for peptic ulcer of the esophagus. Tileston[15] in 1906 suggested that the abuse of alcohol deserves a mention among the predisposing causes, occurring with considerable frequency. Alcohol probably acts in two ways: first, by irritation of the esophagus, and second, by the production of gastritis, with vomiting and insufficiency of the cardia.

Caustic injury by lye

Spechler *et al*[71] reported a case with fundic-type Barrett's epithelium in the mid esophagus with sparing of the distal esophagus complicating caustic injury by lye ingestion.

Cytotoxic chemotherapy agent

Cases of CLE have been reported in 63-75% of patients who received a prolonged course of chemotherapy for breast carcinoma. Sartori *et al*[72] reported that CLE could be acquired through an iatrogenic injury by prolonged chemotherapy with anti-neoplastic agents (cyclophosphamide, methotrexate and 5-fluorouracil). These agents injured the esophageal mucosa and caused columnar epithelium secondary to the drug-induced mucositis. In this regard, they published two reports suggesting that chronic chemotherapy with these agents frequently causes columnar metaplasia of the esophagus. In the first report, they described a woman who, because of duodenal ulcer disease, had an endoscopic examination before the beginning of prolonged chemotherapy for breast cancer. The esophagus was normal, but after 4 mo of chemotherapy, a second endoscopy revealed an esophageal ulcer with columnar epithelium. Endoscopic examinations in another eight women who had been treated with the same chemotherapy agents showed columnar epithelium in six of them. The length of the columnar epithelium was more than 3 cm in all six patients[72]. In the second paper, they reported a prospective endoscopic study carried out to document the development of CLE in women receiving the same chemotherapy drugs**.** Ten of sixteen women (63%) who had no endoscopic or histologic evidence of CLE before chemotherapy were found to develop a CLE after 6 mo of chemotherapy[73].

Acid and pepsin secretion by Barrett's epithelium

Secretion of acid and pepsin by the parietal and chief cells in the Barrett's mucosa has been considered as possible factors in development of CLE by some investigators like Mangla *et al*[30] and Ustach *et al*[74]. However, there are no recent data to support this.

SUMMARY

The incidence of CLE and adenocarcinoma is rising at an epidemic rate, and the cause for this rapid rise is unclear and the answer to this principle question remains to be determined. Due to the causal relationship between CLE and GER, risk factors which enhance reflux such as tobacco smoking, alcohol consumption, dietary changes including high fat diet, obesity, and the wide spread usage of the medications that affect the upper gastrointestinal tract could be partially responsible for this rise. Although other several factors can be offered as possible explanations for this rise in incidence such as increased awareness of the condition, widespread use of endoscopy, and improved diagnostic techniques, this rapid increase in the incidence of CLE and adenocarcinoma raises serious concern that the current management for GER is inadequate and deserves reassessment.

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Reduced expression of β-catenin inhibitor Chibby in colon carcinoma cell lines

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Abstract

AIM: To analyse the Chibby expression and its function in colon carcinoma cell lines and colorectal carcinoma (CRC).

METHODS: Chibby expression levels were investigated by quantitative RT-PCR in a panel of seven different colon carcinoma cell lines. By sequencing, we analysed mutational status of Chibby. To test whether Chibby exhibited effects on β-catenin signalling in colon carcinoma cells, we transfected SW480 cells with Chibby expression plasmid and, subsequently, analysed activity of β-catenin and tested for alterations in cellular phenotype. In addition, we examined Chibby mRNA levels in samples of colorectal carcinomas and adjacent normal tissues by using quantitative RT-PCR and hybridised gene chips with samples from CRC and normal tissues.

RESULTS: Chibby mRNA expression was strongly downregulated in colon carcinoma cell lines in comparison to normal colon epithelial cells and no mutation in any of the examined colon carcinoma cell lines was found. Further, we could show that Chibby inhibited β-catenin activity in TOPflash assays when over-expressed in SW480 cells. Proliferation and invasion assays with Chibby transfected SW480 cells did not reveal profound differences compared to control cells. In contrast to these *in vitro* data, quantitative RT-PCR analyses of Chibby mRNA levels in CRC tumor samples did not show significant differences to specimens in adjacent non-cancerous tissue. Consistent with these findings, gene chips analysing tissue samples of tumors and corresponding normal tissue did not show altered Chibby expression

CONCLUSION: Altered Chibby expression might be observed *in vitro* in different colon carcinoma cell lines. However, this finding could not be confirmed *in vitro* in CRC tumors, indicating that Chibby is not likely to promote CRC tumor development or progression. As Chibby is an important inhibitor of ß-catenin signalling, our data implicate that the usability of colon carcinoma cell lines for *in vitro* studies analysing the Wnt/β-catenin pathway in colorectal carcinoma needs extensive verification.

Key words: Chibby; Wnt; β-catenin; Colorectal carcinoma; Colon carcinoma cell lines

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INTRODUCTION

The Wnt/Wingless pathway has been shown to exhibit important functions in embryonic and cancer development. In the absence of Wnt signalling, β-catenin is phosphorylated by glycogen synthase kinase-3 (GSK-3) and targeted for ubiquitin-mediated proteasomal degradation[1]. Upon Wnt binding to its cellular receptor Frizzled, the degradation complex is destabilised, and, as a result, unphosphorylated β-catenin accumulates and translocates into the nucleus. There, it functions as a co-factor of lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors leading to upregulation of a variety of genes. It has been shown that mutations in different genes of the Wnt pathway are involved in development of various cancers. One of those tumor entities is colorectal carcinoma where approximately 90% of the tumors have been shown to harbour activating mutations within the canonical Wnt signalling pathway[2-4].

The most potent transcriptional activation domain of β-catenin is located within the C-terminal region of β-catenin and has been used as bait in a yeast screening to identify interacting proteins. In this screening, Chibby, a novel protein that is conserved from Drosophila to human, was isolated [5]. It is a nuclear protein of 126 amino

acids, and as it inhibited Wnt signalling by preventing the interaction between β-catenin and LEF/TCF factors, it was suggested as a potential tumor suppressor gene. Chibby is encoded by the human *C22orf2* gene located at chromosome 22q12-q13. This chromosomal region is known to be mutated frequently in colorectal cancer[6], and therefore, Chibby might be an interesting candidate as a tumor suppressor in CRC. Gad *et al*[7] performed loss of heterozygosity (LOH) analyses of tumors of 36 patients involving the *C22orf2* region in CRC and were not able to detect differences between normal and CRC tumor tissues, but did not analysed effects of Chibby on cellular functions.

In this study, we aimed to analyse whether Chibby is mutated in CRC, to evaluate Chibby mRNA expression levels in colon carcinoma cell lines and tumor samples, and to investigate whether Chibby over-expression in colon carcinoma cell lines has an effect on cell behaviour in functional assays.

MATERIALS AND METHODS

Isolation and cultivation of colon epithelial cells

Isolation and cultivation of colon epithelial cells (CEC) were performed as previously described[8,9].

Cell lines and culture conditions

The colon carcinoma cell lines SW48 (ATCC CCL-231), SW480 (ATCC CCL-228), CaCo-2 (ATCC HTB-37), LoVo (ATCC CCL-229), HCT116 (ATCC CCL-247), and HT29 (ATCC HTB-38) were used for *in vitro* experiments. The cell line HT29M3 was chosen as a highly differentiated colon carcinoma cell line[10-13].

For tissue culture, the cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (400 U/mL), streptomycin (50 μg/mL), L-glutamine (300 μg/mL), and 100 mL/L fetal calf serum (FCS) (Sigma, Deisenhofen, Germany) and split 1 : 5 every three days.

For demethylation assays, the cells were treated with 5-azacytidine at a final concentration of 10 μmol/L for 48 h (Sigma)[8]. HT29 M3 cells were used as control cells in the demethylation experiments.

RNA isolation and reverse transcription

For RT-PCR, total cellular RNA was isolated from cultured cells or laser microdissected tissue samples using the RNeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA was controlled on 10 g/L agarose/formaldehyde gel, and subsequently cDNAs were generated by reverse transcriptase reactions. The reverse transcription (RT) reaction was performed in 20 μL reaction volume containing 2 μg of total cellular RNA, 4 μL of 5x first strand buffer (Gibco), 2 μL of 0.1 mol/L DTT, 1 μL of dN_6 primer (10 mmol/L), 1 μL of dNTPs (10 mmol/L) and DEPC water. The reaction mix was incubated for 10 min at 70 ℃. Then 1 μL of Superscript II reverse transcriptase (Gibco) was added and RNAs were reverse transcribed for 1 h at 37 ℃. Subsequently, reverse transcriptase was inactivated at 70 ℃ for 10 min and RNA was degraded by digestion with 1 μL of RNase A (10 mg/mL) at 37 ℃ for 30 min. cDNAs were controlled by PCR amplification of ß-actin.

Chibby mRNA mutational analysis

The entire coding region of Chibby was amplified by RT-PCR from cDNA using16, ATGCCTTTCTTTGGGAATTACGTTC (forward) and chibby496, TCATTTTCTCTTCCGGCTGATC (reverse), which resulted in a 380-bp fragment. The PCR reaction was performed in 50 μL reaction volume containing 5 μL of 10x Taq buffer, 1 μL of cDNA, 0.5 μL of each primer (0.2 μmol/L), 0.5 μL of dNTPs (10 mmol/L), 0.5 μL of Taq polymerase (5 U/μL), and 41 μL of water. The amplification reactions were performed by 35 repetitive cycles of denaturation for 30 s at 94 ℃, annealing for 30 s at 64 ℃, extension for 30 s at 72 ℃, and a final extension step at 72 ℃ for 5 min. The PCR products were resolved on 15 g/L agarose gels. For sequencing, the products were purified by PEG precipitation to remove unincorporated primers and dNTPs. Both strands were sequenced for each PCR product from at least two independent PCR reactions. Sequences were compared with the gene data bank by means of BLAST search (National Center of Biotechnology Information).

Analysis of Chibby expression by quantitative PCR

Quantitative real-time PCR was performed on a LightCycler (Roche, Mannheim, Germany). Two microliters of cDNA template, 1.6 μL of 25 mmol/L $MgCl_2$, 0.2 μmol/L forward and reverse primers {chibby125, TTTGGGAATACGTTCAGTCCG (forward) and chibby395, TCAGCCGCAAGAGATTGTTC (reverse)} and 2 μL of SybrGreen LightCycler mix in 20 μL volume were PCR-amplified through 40 cycles, each cycle consisting of initial denaturation at 95 ℃ for 30 s, 20 ℃/s temperature transition rate up to 95 ℃ for 15 s, 64 ℃ for 3 s, 72 ℃ for 5s, 85 ℃ acquisition mode single. The PCR product was evaluated by melting curve analysis following the manufacturer's instructions and by checking the PCR products on 18 g/L agarose gels. All quantitative PCR experiments were repeated three times.

Transient transfection of SW480 cells

A total of 2×10^5 cells were seeded onto six-well plates one day before transfection. Transfections with pCMX PL2 or pCMX PL2 flag Chibby plasmid (0.5 μg per well) were performed using the LipofectAMINE plus method (Life Technologies) according to the manufacturer's instructions. Cells were harvested 48 h after transfection and the amount of Chibby expression was determined by RT-PCR and Western blot analysis.

Protein analysis in vitro (Western-blotting)

For protein isolation, 2×10^6 cells were washed in 1x PBS and lysed in 200 μL RIPA buffer (Roche). The protein concentration was determined using the BCA protein assay reagent (Pierce, USA). Equal amounts of cellular protein (40 μg total protein) were denatured at 94 ℃ for 10 min after addition of Rotiload buffer (Roth, Karlsruhe, Germany) and subsequently, separated on NuPage SDS gels (Invitrogen, Groningen, The Netherlands). After transferring the proteins onto PVDF membranes (BioRad, Richmond,

USA), the membranes were blocked in 50 g/L dry milk in TBS-T and incubated overnight with primary monoclonal anti flag antibody (1 : 4500 dilution, Sigma) at 4 ℃. A 1 : 4 000 dilution of anti IgG AP (Sigma) was used as secondary antibody. Staining was performed using 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium tablets (Sigma). All Western blot experiments were repeated at least three times.

Immunofluorescence staining of flag-tagged Chibby

A total of 2×10^4 SW480 cells were seeded per chamber of four-well-Falcon chamber slides (Becton Dickinson, Le Pont de Plaix, France) and cultivated overnight before transfection with either control vector or Chibby flag pCMX PL2. After 48 h of transfection, cells were washed with PBS, fixed with 40 g/L paraformaldehyde, permeabelised with 1 ml/L Triton X-100, washed again, and blocked for 30 min in 10 g/L BSA in PBS. Anti flag antibody (dilution 1 : 1 000) was used as primary antibody, and anti mouse FITC-conjugated antibody (dilution 1 : 40) was used as secondary antibody. Incubations took place at room temperature for 1 h each. Staining of nuclei occurred with DAPI.

Reporter gene assays

For transient transfections, 2×10^5 cells per well were seeded into six-well plates and transfected with 0.5 μg of TOPflash or FOPflash reporter gene plasmids using the LipofectAMINE plus method (Gibco) according to the manufacturer's instructions. For cotransfection, 0.125 μg, 0.25 μg, 0.5 μg, and 1 μg of Chibby expression plasmid per well were used, respectively. At 48 h post-transfection, the cells were lysed and the luciferase activity in the lysate was measured. To normalise transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega, Mannheim, Germany) was cotransfected and renilla luciferase activity was measured by a luminometric assay (Promega). All transfection experiments were repeated at least three times.

Proliferation assay

Proliferation was measured as previously described[14]. Proliferation of Chibby transfected SW480 cells was compared to non or mock transfected SW480 cells in an XTT assay. Results were expressed as mean ± SD (range). All experiments were repeated three times.

Invasion assay

Invasion assays were performed as previouly described[14]. Briefly, the assays were performed in Boyden chambers containing polycarbonate filters with an 8-μm pore size. In brief, filters were coated with a commercially available reconstituted basement membrane (Matrigel; Becton Dickinson, Heidelberg, Germany), and the lower compartment was filled with fibroblast-conditioned medium as chemoattractant. SW480 cells and Chibby transfected SW480 cells were harvested by trypsinization, resuspended in DMEM without FCS at a density of 2×10^5 cells/mL, and placed in the upper compartment of the chambers. After incubation for 4 h at 37 ℃, the filters were removed. The cells adhering to the lower surface were fixed, stained, and counted.

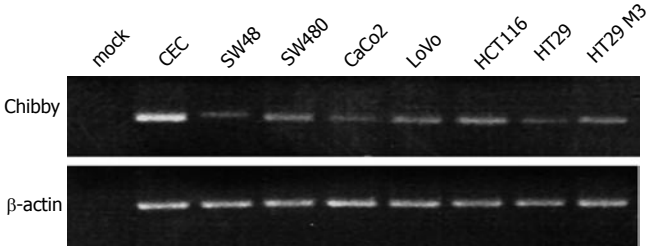

Figure 1 PCR amplification of the complete coding region of Chibby mRNA. The Chibby coding region was amplified using RT-PCR. All colon carcinoma cell lines were shown to express full-length Chibby mRNA. Expression levels cannot be estimated from this experiment as the PCR was carried out to saturation in order to illustrate full-length mRNA expression and to obtain enough PCR products for sequencing. β-actin amplification served as control to ascertain the integrity of the cDNA in all samples.

Fifteen random fields were counted at 200-fold magnification. Each sample was assayed in triplicate, and all experiments were repeated three times.

RESULTS

It is well known that the Wnt/β-catenin signalling pathway is constitutively active in a large number of colorectal cancers. The most commonly mutated proteins within the pathway are β-catenin and APC. Intriguingly, the activation of the cascade cannot in all cases of CRC be explained by mutations in these proteins. It remains unclear what other proteins of the pathway have to be altered in addition to the aforementioned mutations to lead to activation of the Wnt/β-catenin signalling pathway. Hence, we were interested in analysing other potential endogenous inhibitory factors that might accomplish regulatory functions within the Wnt/β-catenin signalling pathway and thus influence signal transduction. Due to these reasons, we analysed the role of the β-catenin-associated antagonist Chibby in colorectal carcinoma cell lines and compared our *in vitro* findings of Chibby expression with the *in vivo* situation by analysing colorectal tumor samples and the corresponding normal tissue.

Analysis of Chibby mutational status and expression level in colon carcinoma cells

We analysed a panel of seven colon carcinoma cell lines, such as SW48, SW480, CaCo2, LoVo, HCT116, HT29, and HT29 M3, that are frequently used as model systems to study effects in colorectal carcinoma. We evaluated the panel of colon carcinoma cell lines for alterations in the Chibby mRNA expression. The complete Chibby coding region was amplified by RT-PCR. All cell lines examined expressed Chibby mRNA at the expected length, indicating that no deletions or mutational insertions had occurred (Figure 1). As a control the Chibby coding sequence was amplified using CEC cDNA as template. The same cDNAs were used to amplify β-actin as a control for the integrity of the cDNA.

The cell lines were evaluated for levels of Chibby mRNA expression using quantitative RT-PCR in comparison to normal colon epithelial cells (CEC). Chibby

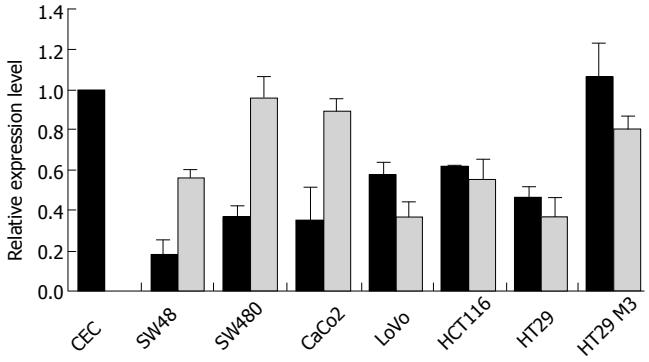

Figure 2 Quantification of Chibby expression in colon carcinoma cell lines. The amount of Chibby mRNA expression was carefully quantified by real-time PCR. All colon carcinoma cell lines showed down-regulation of Chibby mRNA expression in comparison to colon epithelial cells (CEC) and HT29 M3 cells, a cell line derived from HT29 cells that is considered to be a highly differentiated colon carcinoma cell line (black bars). Treatment of cells with the demethylating agent 5-azacytidine caused a modest increase in Chibby mRNA levels in SW48, SW480, and CaCo2 cells, but not in the other cell lines (grey bars). HT29 M3 cells were used as controls for the demethylation experiments as the experiment could not have been performed with primary CEC due to culture conditions.

mRNA was found to be down-regulated in all cell lines in comparison to CEC and HT29 M3 cells which are considered to be a highly differentiated colon carcinoma cells and express Chibby in amounts similar to those of CEC (Figure 2, black bars). Three of the analysed cell lines, SW48, SW480, and CaCo2 cells, showed residual expressions of Chibby mRNA of 18%, 37%, and 35%, respectively, in comparison to CEC or HT29 M3 (100% and 106%, respectively). LoVo, HCT116, and HT29 cells were also shown to have reduced Chibby mRNA levels, but down-regulation was not as explicit as in the other examined cell lines (57%, 62%, and 46%).

To investigate the reason for down-regulation of Chibby expression in colon carcinoma cell lines, we amplified and analysed the exons of the *chibby* gene. All PCR products were purified and subsequently sequenced using two different primers. The analysis of sequenced PCR products did not reveal any sequence variation within the coding region of the *chibby* gene locus (data not shown).

From these results, we concluded that Chibby does not harbour mutations within the coding sequence in all seven analysed colon carcinoma cell lines.

As this reduction in mRNA expression in colon carcinoma cell lines could not be explained by loss or mutation of the *chibby* gene, we hypothesised that promoter hypermethylation could have silenced gene expression. To test this hypothesis, all cell lines were exposed to the demethylating agent 5-azacytidine. Treated and untreated cells were tested for Chibby mRNA expression which was quantified by quantitative RT-PCR. HT29 M3 cells were used as controls in these experiments. HT29 M3 cells are highly differentiated cells that were used as an additional control as CEC could not be used in demethylation experiments as controls due to culturing issues.

Our results showed that 5-azacytidine treatment did not lead to induction of Chibby expression in LoVo, HCT116, HT29, or HT29 M3 cells, but to a moderately increased expression in SW48, SW480, and CaCo2 cells (Figure 2, grey bars). Therefore, we conclude that promoter hypermethylation might play a minor role for altered Chibby expression in some of the analysed cell lines, but we do not consider this silencing mechanism to be a general event in down-regulation of Chibby mRNA expression in the colon carcinoma cell lines.

Effect of Chibby expression on β-catenin transcriptional activity

As colon carcinoma cell lines display reduced levels of Chibby expression in comparison to normal CEC, we were interested in whether over-expression of Chibby in these cells influences functional processes. To test for the potential effects, we over-expressed Chibby in the colon carcinoma cell line SW480 by transfecting a flag-tagged Chibby expression construct. Successful over-expression was observed by quantitative RT-PCR (data not shown) and Western blotting (Figure 3A). Exogenous Chibby protein was not detected following mock or control transfection with pCMX PL2 flag plasmid which did not alter Chibby levels. Strong over-expression of Chibby was observed after transfection of SW480 cells with Chibby flag expression plasmid. Immunofluorescence staining revealed correct expression patterns of Chibby flag-tagged protein in the nucleus of SW480 cells (Figure 3B), also displaying high transfection efficiency.

As Chibby as endogenous antagonist of β-catenin is expected to exert negative functions on the Wnt/β-catenin signalling pathway, we performed LEF/TCF luciferase reporter gene assays using the TOPflash/FOPflash system. Analysing the activity of β-catenin in control and Chibby over-expressing cells, we observed reduced activation levels in the TOPflash system in accordance to the amount of transfected Chibby expression plasmid (Figure 3C). Transfection with 0.125 μg or 0.25 μg of Chibby plasmid per well did not affect the activity of TOPflash reporter, whereas activity dropped to 44% and 48%, respectively, when 0.5 μg and 1 μg expression plasmids were applied. FOPflash reporter construct activity was not affected by Chibby expression (Figure 3C). These results showed that transfection of SW480 cells with Chibby expression plasmid reveals functional Chibby protein.

Functional assays with Chibby over-expressing SW480 cells

To assay whether altered Chibby levels had an impact on proliferation and invasion potential of SW480 cells, we performed functional experiments utilizing mock and Chibby transfected cells. Boyden chamber assays were performed to examine the effect of Chibby on the invasiveness of SW480 cells. In this assay, the tumor cells have to invade through matrigel mimicking the basal lamina. Comparing mock treated cells, control transfected cells, and Chibby over-expressing cells, we did not observe any differences with regard to their invasiveness.

As the Wnt/β-catenin signalling pathway is known to influence cell proliferation, we next assayed for alterations of proliferation and performed *in vitro* proliferation assays with Chibby transfected SW480 cells and mock or control transfected cells. Modifying the Chibby protein level had

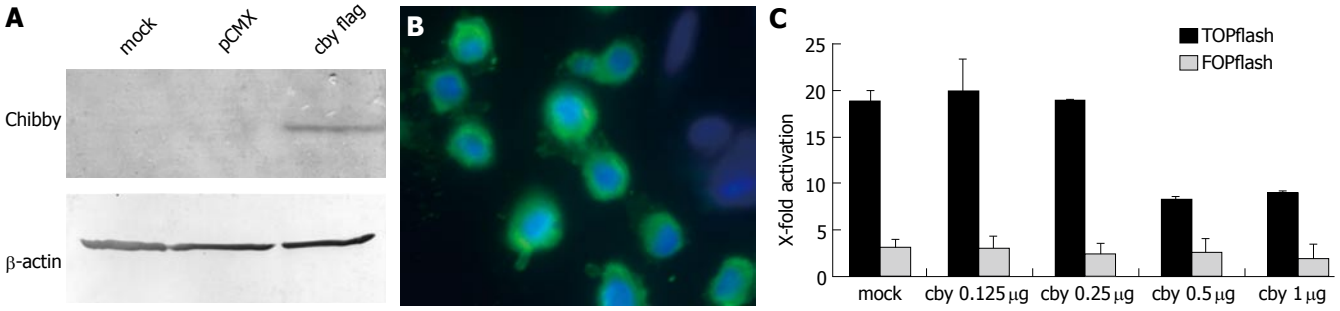

Figure 3 Expression of Chibby in transfected SW480 cells. A: Western blot analysis showing Chibby protein expression levels in untransfected (mock), control vector, or Chibby transfected SW480 cells. B: immunofluorescence staining of flag-tagged Chibby transfected SW480 cells. Nuclei were stained with DAPI in blue, flag-tagged Chibby was detected with FITC-labelled anti-flag antibody (green). C: TOPflash reporter gene assays showing inhibition of β-catenin signalling after transfection of SW480 cells with Chibby expression plasmid.

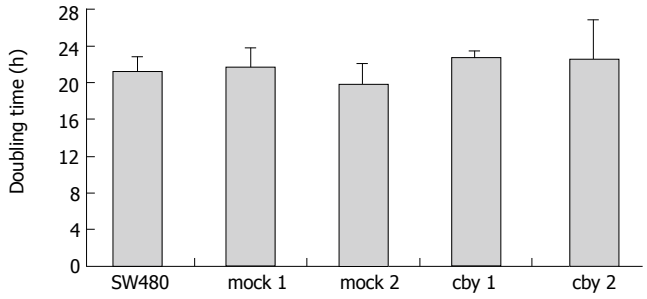

Figure 4 Proliferation Assay. Analysis of the cells over-expressing Chibby could not altered doubling time in comparison to mock or control transfected SW480 cells. Assays were performed in triplicates.

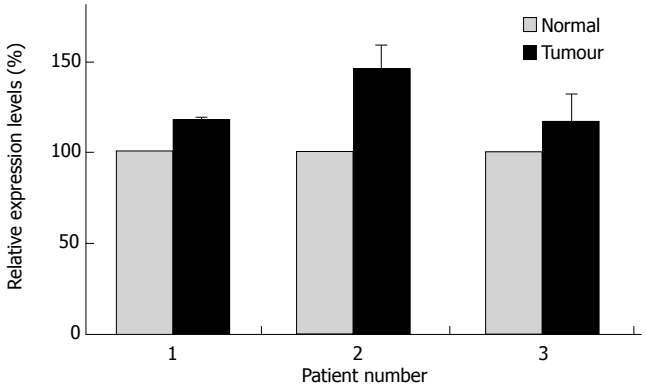

Figure 5 Chibby mRNA expression levels in colorectal tumor and corresponding normal tissue samples. Quantitative RT-PCR showing Chibby mRNA expression levels in matched pairs of tumor and normal colorectal tissue of 3 different patients.

no significant effect on cell proliferation (Figure 4). Doubling times were as follows: SW480: 21.2 h, mock 1: 21.7 h, mock 2: 19.7 h, cby 1: 22.6 h, and cby 2: 22.5 h, respectively. Thus, we did not consider proliferation to be affected by Chibby expression levels in SW480 cells.

Chibby mRNA expression in vivo

As our experiments in colon carcinoma cell lines had revealed sound down-regulation of Chibby expression, the absence of effects on cell functions of over-expressed functional Chibby protein in one of these cell lines (SW480) was rather unexpected. We next performed experiments with samples from patients with diagnosed colorectal carcinoma to assess the question whether Chibby expression was altered not only in colon carcinoma cell lines, but also in coloreactal tumors and might therefore contribute to or support development and progression of colorectal tumors.

We analysed Chibby transcript expression levels in tumor samples and corresponding normal colonical tissue from the same patients by hybridisation of an oligonucleotide-based array (Affymetrix HG-U133A) with 43 samples of colorectal cancer and 34 matched normal mucosa (unpublished data). The ratio of expression levels between tumors and normal tissues was 1.11, displaying no altered Chibby expression in CRC tumors in comparison to adjacent normal colon tissue. In addition, when tumor samples with microsatellite stability (MSS; $n = 24$) and microsatellite instability (MSI; $n = 19$) were separately assessed, again no difference in Chibby expression was observed.

To further validate this result, using quantitative RT-PCR, we tested whether expression levels of Chibby mRNA differed between normal colorectal tissue and adjacent cancerous tissue. We manually microdissected frozen material and analysed the extracted mRNAs. Figure 5 shows the results of 3 examined matched samples, displaying no profound alterations between tumor and adjacent normal tissue. Relative Chibby levels ranged from 100% in normal tissue to 117.4% (patient 1), 146.1% (patient 2), and 116.4% (patient 3), respectively.

DISCUSSION

The cellular antagonist and β-catenin interacting factor Chibby, a small nuclear protein, has been shown to be an endogenous inhibitor of the canonical Wnt signalling pathway. It binds to the LEF/TCF interaction domain at the C-terminus of β-catenin and thereby eliminates interaction of β-catenin with LEF/TCF transcription factors[5]. This leads to a block of transcriptional activation within the signalling cascade. It has been shown for colorectal carcinoma that more than 90% of all tumors harbour activating mutations within proteins of the canonical Wnt/β-catenin signalling cascade[2] and most mutations affect either the β-catenin or the APC

protein[14,15]. Interestingly, tumor development cannot in all cases be solely explained by these mutations, but there need to be further and additional disrupting cellular events. Hence, we were analysing other factors within the Wnt/β-catenin pathway and this study showed our results on the cellular antagonist of β-catenin, Chibby. Due to its function, Chibby has been suggested as a potential tumor suppressor[5]. The aim of our study was to analyse whether Chibby expression is altered in colon carcinoma cell lines and tumors, and if so, whether restoration of Chibby amounts in colon carcinoma cell lines affects cell functions and behaviour.

Examining six colon carcinoma cell lines and one highly differentiated cell line (HT29 M3) that was used as additional control with regard to the mutational status, we could not detect any alterations within Chibby coding sequence. Using quantitative RT-PCR methods, we detected down-regulation of Chibby mRNA expression levels in all six cell lines in comparison to normal colon cells (CEC) or HT29 M3. We demonstrated in another study that gene silencing via promoter hypermethylation might be responsible for down-regulated transcript expression if the coding region does not harbour any mutations[8]. We, therefore, speculated that promoter hypermethylation might be a mechanism for Chibby down-regulation in the examined cell lines, but treating cells with the demethylating agent 5-azacytidine did not lead to sound induction of Chibby mRNA expression in all cell lines. SW48, SW480, and CaCo2 cells showed the lowest default expression levels among all the cell lines tested and displayed a moderate increase in Chibby mRNA expression upon 5-azacytidine treatment. But we do not reckon epigenetic alterations as a common mechanism for Chibby down-regulation.

To assess the functional implication of reduction of chibby expression, we used the cell line SW480 to re-express Chibby via transfection of a Chibby expression plasmid. Tests for correct expression of Chibby were followed by functional assays for proliferation and invasion. We were not able to detect altered invasion potential of Chibby over-expressing cells in comparison to control cells or mock transfected cells. Also, proliferation rate of control and Chibby transfected cells was not different (Figure 4) by means of doubling times. These findings were unexpected as Chibby seemed to be a promising candidate as tumor suppressor in colorectal carcinoma. We speculate that the experimental over-expression of Chibby in SW480 cells does not lead to changes in cell behaviour as mutations in other molecules further downstream within the cascade could overcome Chibby effects. This hypothesis is based on the knowledge that the SW480 cell line is known to harbour several mutations, not only in the Wnt/β-catenin signalling pathway but also in a number of other proto-oncogenes[16,17].

In the very contrast to our findings concerning Chibby expression in a panel of colon carcinoma cell lines, the *in situ* and *in vivo* analyses of colorectal tumors did not support the *in vitro* conclusions. Neither quantitative RT-PCRs, nor hybridisation of an oligonucleotide-based array gave an indication of dysregulation of Chibby in colorectal tumors in comparison to normal colorectal tissue. Our results are supported by a recent study by Gad *et al*[17] who have reported neither somatic mutations in samples of colorectal tumors from 36 patients, nor have they been able to correlate Chibby expression levels to tumorigenicity. Gad *et al*[17] have reported very similar findings to our data that also display no differences in expression levels between normal colon tissue and tumor material.

Taking these data together, we conclude that in contrast to colon carcinoma cell lines where Chibby expression was down-regulated in comparison to normal colonical epithelial cells, *in vivo* Chibby expression is not altered in colorectal tumors and therefore neither a cause nor an indicator for tumor development in colorectal carcinoma.

Our data point out that the use of colon carcinoma cell lines to analyse events within the Wnt/β-catenin signalling pathway has to be considered very carefully. The status of protein expression in these cell lines, at least with regard to Chibby, varies compared to that of tumors and may end up in different phenotypical behaviour of the cells. Thus, direct conclusions on *in vivo* situations could be misleading. Therefore, these differences have to be taken into account when performing *in vitro* studies on cellular signalling pathways.

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COLORECTAL CANCER

Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry

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Abstract

AIM: To detect the new serum biomarkers for colorectal cancer (CRC) by serum protein profiling with surface-enhanced laser desorption ionisation - time of flight mass spectrometry (SELDI-TOF MS).

METHODS: Two independent serum sample sets were analysed separately with the ProteinChip technology (set A: 40 CRC + 49 healthy controls; set B: 37 CRC + 31 healthy controls), using chips with a weak cation exchange moiety and buffer pH 5. Discriminative power of differentially expressed proteins was assessed with a classification tree algorithm. Sensitivities and specificities of the generated classification trees were obtained by blindly applying data from set A to the generated trees from set B and vice versa. CRC serum protein profiles were also compared with those from breast, ovarian, prostate, and non-small cell lung cancer.

RESULTS: Mass-to-charge ratios (m/z) 3.1×10^3, 3.3×10^3, 4.5×10^3, 6.6×10^3 and 28×10^3 were used as classifiers in the best-performing classification trees. Tree sensitivities and specificities were between 65% and 90%. Most of these discriminative m/z values were also different in the other tumour types investigated. M/z 3.3×10^3, main classifier in most trees, was a doubly charged form of the 6.6×10^3-Da protein. The latter was identified as apolipoprotein C-I. M/z 3.1×10^3 was identified as an N-terminal fragment of albumin, and m/z 28×10^3 as apolipoprotein A-I.

CONCLUSION: SELDI-TOF MS followed by classification tree pattern analysis is a suitable technique for finding new serum markers for CRC. Biomarkers can be identified and reproducibly detected in independent sample sets with high sensitivities and specificities. Although not specific for CRC, these biomarkers have a potential role in disease and treatment monitoring.

Key words: Proteomics; Colorectal cancer; Biomarker; Sensitivity; Specificity

Engwegen JYMN, Helgason HH, Cats A, Harris N, Bonfrer JMG, Schellens JHM, Beijnen JH. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. *World J Gastroenterol* 2006; 12(10): 1536-1544

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INTRODUCTION

Colorectal cancer is the third most common cause of cancer-related death in both men and women, accounting for about 10% of all cancer deaths annually. When diagnosed and treated early, the overall 5-year survival rate is around 90%. However, most patients present with locally advanced or metastasised disease at the time of diagnosis, or develop metastasis during follow-up. Suitable tumour markers will facilitate colorectal cancer detection, determination of prognosis, and disease and therapy evaluation.

However, currently used non-invasive methods, such as measurement of serum carcinoembryonic antigen (CEA) levels, faecal occult blood testing and faecal DNA analysis,

have low sensitivities and/or specificities for colorectal cancer[1-4]. Although CEA is currently the best available marker for follow-up of resected colorectal cancer and monitoring of chemotherapy, its use to determine eligibility for adjuvant therapy or its routine use as a single parameter for treatment monitoring has significant clinical limitations[5,6].

Detection of so-called biomarker proteins in serum may lead to new and better tumour markers for colorectal cancer. The proteome, contrary to the genome, is not a static parameter: it reflects not only the presence of active or inactive (mutated) genes, but also their extent of expression at a specific time point. In addition, the proteome reflects all proteins and peptides that may rise from only one gene, i.e. different cleavage products and proteins with different post-translational modifications. Both characteristics allow a more detailed evaluation of a disease status using the human proteome.

Protein profiling in complex biological matrices has become more easily achievable with the Surface-Enhanced Laser Desorption Ionisation (SELDI) ProteinChip technology in combination with a time of flight (TOF) mass spectrometer. This is a relatively new technique, which lacks the disadvantages of 2D-gel-electrophoresis for proteomic research in that it has high sensitivity in the low molecular weight range and high throughput capability, and proteins with extreme characteristics (highly hydrophobic, acidic or basic) can be analysed more easily[7,8]. With this technique, whole serum is applied to protein chips with different chromatographic affinities in a suitable binding buffer. Selectively bound proteins are retained on the surface and non-selectively bound proteins are washed off. In the mass spectrometer, a laser desorbs the bound proteins from the chip surface, which are subsequently detected in the TOF analyser by their respective mass-to-charge ratios (m/z). Since a whole pattern of proteins is analysed, more than one biomarker can be detected. Combination of several of these biomarkers for the evaluation of a patient's status may result in enhanced sensitivities and specificities.

SELDI-TOF MS has already been applied to several forms of cancer, including breast, ovarian, prostate, and lung cancer[9-12]. In the obtained protein profiles, proteins with high sensitivity and specificity for disease have been detected. For colorectal cancer, a discriminative protein of $12*10^3$ Da has been found in tumour cell lines, the identity of which was prothymosin-alpha[13]. Comparing epithelial colorectal carcinoma cells with normal tissue, 3.48×10^3-, 3.55×10^3- and 3.6×10^3-Da proteins were found to be increased in cancer tissue[14]. In Asian patients with colorectal cancer and healthy controls, discriminating serum protein profiles have been recently reported, m/z 5911, 8922, 8944 and 8817 being the most important biomarkers[15-18]. These results were obtained in a single sample set and the reported sensitivities and specificities resulted from cross-validation within this single set. In addition, the identities of the reported biomarker proteins remain unknown.

The objective of this study was to detect biomarker proteins for colorectal cancer in serum using SELDI-TOF MS, and to validate these with an independent sample set. In addition, we aimed at identifying any found biomarkers so that further insight into the pathological processes involved in colorectal cancer can be obtained.

MATERIALS AND METHODS

Patient samples

Two independent serum sample sets were analysed for their protein profiles on different occasions. The first set consisted of samples from 40 patients with colorectal cancer (all Dukes' D) and 49 healthy controls. The second set consisted of samples from 37 patients with colorectal cancer (1 Dukes' A, 2 Dukes' B, 12 Dukes' C, 17 Dukes' D, 5 unknown) and 31 healthy controls. For comparison of colorectal cancer protein profiles with those from other tumour types, a third sample set consisting of serum samples from 8 non-small cell lung cancer (NSCLC) patients (stage III and IV), 10 breast cancer patients (stage II and III), 10 prostate cancer patients (stage I-IV), and 10 ovarian cancer patients (stage I-IV) was analysed. All serum samples were obtained from the serum bank at the Netherlands Cancer Institute, where they were stored at -30 ℃ until analysis. Sample collection was performed after taking individuals' informed consent under approval of the Institutional Review Board. Samples were drawn before surgery or chemotherapy was started, except for 9 patients with metastatic disease in sample set B who had already had surgery.

Protein profiling

Protein profiling was performed using SELDI-TOF MS (Ciphergen Biosystems Inc., Freemont, CA, USA). Several chromatographic chip surfaces and binding conditions were screened for discriminative m/z values between colorectal cancer patients and healthy controls. The most discriminating peaks were seen on CM10 chips, a weak cation exchange chip, which contains anionic carboxylate groups that bind positively charged proteins in serum. Best results were obtained using a sodium phosphate binding buffer (pH 5) and a 500 mL/L solution of sinapinic acid (SPA; Ciphergen Biosystems) in 500 mL/L acetonitrile (ACN) + 5 mL/L trifluoracetic acid (TFA) as energy absorbing molecule.

All serum samples were denatured by adding 180 μL of 9 mol/L urea, 20 g/L CHAPS, 10 g/L DTT (all from Sigma, St. Louis, MO, USA) to 20 μL of serum. CM10 chips were assembled in a 96-well format bioprocessor (Ciphergen Biosystems) which can hold twelve 8-spot protein chips. During all steps of the protocol, the bioprocessor was placed on a platform shaker at 350 r/m. Chips were equilibrated twice with 200 μL of binding buffer consisting of 20 mmol/L sodium phosphate (Sigma) buffer (pH 5) with 1 g/L TritonX-100 (Sigma) for 5 min. Subsequently, 180 μL of binding buffer and 20 μL of denatured sample were applied to the chip. Sample allocation was at random. Incubation was set to 30 min. After binding, the chips were washed twice for 5 min with binding buffer, followed by two 5-min washes with binding buffer without TritonX-100. Lastly, chips were rinsed with deionised water. After air-drying, two times 1 μL of the SPA was applied to the spots.

Protein chips were analysed using the PBS-IIC

ProteinChip Reader (Ciphergen Biosystems). Data were collected between 0 and 200 000 Da. Data collection was optimised for detection of discriminating peaks, resulting in an average of 65 laser shots per spectrum at laser intensity 150 and detector sensitivity 8, and laser focusing at 3000 Da. M/z values for the detected proteins were calibrated externally with a standard peptide mixture (Ciphergen Biosystems) containing [Arg8] vasopressin (1 084.3 Da), somatostatin (1 637.9 Da), dynorphine (2 147.5 Da), ACTH (2 933.5 Da), insulin β-chain (bovine) (3 495.9 Da), insulin (human recombinant) (5 807.7 Da), and hirudin (7 033.6 Da).

Statistics and bioinformatics

Data were analysed with ProteinChip Software package, version 3.1 (Ciphergen Biosystems). For each sample set, all acquired spectra were compiled and analysed as a whole. Spectra were baseline subtracted and normalised to the total ion current between 1 500 and 200 000 Da. For validation of either sample set, the normalisation factor from the training set was applied to the spectra of the validation set. The Biomarker Wizard (BMW) software application (Ciphergen Biosystems) was used to autodetect m/z peaks with a signal-to-noise ratio of at least 5. Peak clusters were completed with peaks with a signal-to-noise ratio of at least 2 in a 0.5% cluster mass window. For validation purposes, peak clusters of the training set were applied in the validation set. Group differences were calculated with the same application, comparing mean intensities of all detected peaks between groups with non-parametric statistical tests. *P* values less than 0.01 were considered statistically significant.

Next, Biomarker Patterns Software (BPS; Ciphergen Biosystems) was used to generate classification trees from the BMW files. A classification tree is built of nodes with an m/z value and a cut-off value for the peak intensity. An example of such a tree is shown in Figure 1. When an analysed spectrum has a peak intensity at the specified m/z below the cut-off value, the sample is placed in the left tree branch. Otherwise, it is placed to the right and its peak intensity at the next m/z value is evaluated. Peaks that result in a maximum separation of the two groups, with a minimum of misclassification are chosen for the nodes. The branch consists of new nodes with an m/z value until a final classification can be made for the spectrum: originating from a colorectal cancer patient or from a healthy control. For every tree, the BPS performs a ten-fold cross-validation in the tree building process, in which ten times another tenth of the data set is used for testing of the tree and these results are combined to yield a cross-validation sensitivity and specificity as a measure for the tree's discriminative power. However, to obtain a more realistic sensitivity and specificity, classification trees built with one sample set as the training set were validated with the blinded data from the remaining set. In addition, both sample sets were combined to form a training set with two thirds of all samples keeping a random third behind in the tree building process for independent validation afterwards.

Biomarker purification and identification

Biomarkers detected in the profiling experiments were purified by fractionation of denatured serum on QhyperD beads (Biosepra; Ciphergen Biosystems), a strong anion exchange surface, with decreasing buffer pH. Subsequently, fractions containing the markers were concentrated on microcon YM-50 filters (Millipore, Billerica, MA, USA) and eluted with increasing concentrations of ACN + TFA (1: 0.001, v/v). The purification process was monitored by profiling each fraction on NP20 chips, containing a non-selective, silica chromatographic surface. Relevant eluates were evaporated and redissolved in loading buffer for SDS-PAGE. Gel electrophoresis was performed on Novex NuPage gels (Invitrogen, San Diego, CA, USA). Gels were stained using colloidal Coomassie staining (Simply Blue; Invitrogen) and protein bands of interest were excised and collected for either passive elution, followed by in-solution digestion or in-gel digestion with trypsin (Promega, Madison, WI, USA).

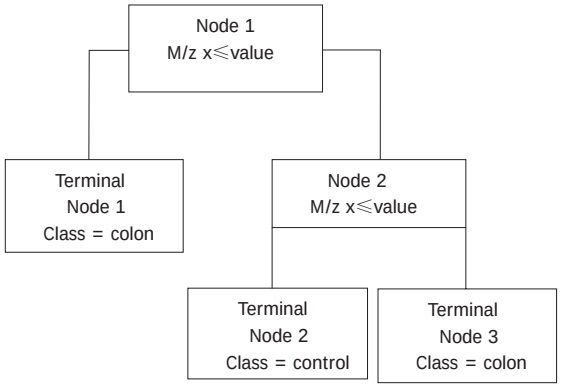


Figure 1 Example of a BPS-generated classification tree distinguishing colorectal cancer patients and healthy controls. If the peak intensity of an analyzed sample is below the cut-off value at the m/z in the node, the sample proceeds to the left. If not, it proceeds to the right, where its peak intensity at the next m/z is evaluated.

For passive elution, bands were washed twice with 300 mL/L ACN + 100 mmol/L NH_4HCO_3 (Sigma), followed by dehydration in ACN. Samples were heated at 50 °C and then eluted with 15 μL of formic acid/ACN/isopropanol/deionised water (4.5:3:1:1.5, v/v) under sonification for 30 min. The eluate was left for 3 h at room temperature before profiling on NP20 chips. Then, the eluate was left overnight. In-solution digests were obtained by evaporation of the supernatant in a SpeedVac, resuspending it in 20 mg/L trypsin in 100 mL/L ACN + 25 mmol/L NH_4HCO_3 and incubation for 4 h at room temperature.

In-gel digestion was performed after washing the excised band with methanol/acetic acid/deionised water (4:1:5, v/v) twice, followed by a wash with 300 mL/L ACN + 100 mmol/L NH_4HCO_3. Samples were dried on a SpeedVac and immersed in a 20 mg/L-solution of trypsin in 100 mmol/L NH_4HCO_3. Digestion was allowed for 12 h at room temperature. All tryptic digests were profiled on NP20 chips, using 1 μL 200 g/L CHCA (Ciphergen Biosystems) solution in 500 mL/L ACN + 5 mL/L TFA as matrix.

Peptides in the resulting digest were investigated with the MASCOT and ProFound search engines (http://www.matrixscience.com; http://prowl.rockefeller.edu/profound_bin/WebProFound.exe), using the Swiss-Prot

Table 1 Classification trees generated with the Biomarker Patterns Software

Tree	Included m/z's ($\times 10^3$ Da): cut-off intensity values and class assignment				Sensitivity (%)	Specificity (%)
	Node 1	Node 2	Node 3	Node 4		
I	3.3 ⩽ 15.035	-	-	-	77.8	73.3
	Colorectal cancer					
II	28 ⩽ 1.558	4.5 ⩽ 29.791	3.1 ⩽ 9.866	6.6 ⩽ 33.233	77.8	73.3
	Colorectal cancer	to Node 3	to Node 4	Colorectal cancer		
III	3.3 ⩽ 12.757	-	-	-	66.7	83.3
	Colorectal cancer					
IV	3.3 ⩽ 12.757	28 ⩽ 1.285	-	-	75.0	83.3
	Colorectal cancer	Colorectal cancer				
V	3.3 ⩽ 12.981	4.5 ⩽ 28.599	-	-	84.2	83.3
	Colorectal cancer	Healthy control				
VI	28 ⩽ 1.529	4.5 ⩽ 28.577	6.6 ⩽ 44.685	-	89.5	88.9
	Colorectal cancer	to Node 3	Colorectal cancer			

Sensitivities and specificities for trees I to IV were obtained by blinded testing with data from the remaining sample set.

and NCBI databases, respectively. Data were searched against the Homo sapiens subset of the database, defining fixed modification of the cysteine residues with propionamide and variable modification of methionine residues (oxidation). Peptide mass tolerance of the average MH^+ masses was 0.5 - 3 Da; the number of tryptic miscleavages allowed was 1 or 2.

Identification of proteins was confirmed either by immunoassay on protein A beads (Biosepra; Ciphergen Biosystems) with an appropriate antibody (Abcam Ltd, Cambridge, UK), or by sequencing of the most important peptides in the tryptic digest with tandem MS on both a Q-TOF™ II, (Micromass Ltd, UK) and a QSTAR ™ (AB/Sciex, Foster City, CA, USA), both equipped with a PCI 1000 interface (Ciphergen Biosystems). For the immunoassay, beads were loaded with antibody in phosphate-buffered saline (PBS, Sigma), and washed twice with PBS, followed by a 30-min incubation with whole serum, 5 subsequent washes with PBS and one with deionised water. Finally, bound proteins were eluted using 1 mol/L acetic acid and the eluate was profiled on NP20 chips.

Serum CRP, TRF and CEA quantification

Serum CEA was quantified using an electrochemiluminiscence immunoassay on a Modular analytics E170 analyser (Roche Diagnostics, Mannheim, Germany). A cut-off value of 5 μg/L was employed. Levels of the acute phase reactants C-reactive protein (CRP) and transferrin (TRF) were assessed by a near infrared particle immunoassay and a turbidimetric immunoassay, respectively, using the Beckman Synchron LX20 analyser (Beckman-Coulter Inc., Fullerton, CA, USA). CRP levels below 8 mg/L were considered clinically normal, as are TRF levels between 2.1 and 3.8 g/L. All statistical analyses for these data were performed with SPSS, version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Biomarker detection

In the first and second sample set respectively, 15 and 6 proteins of which the expression differed in colorectal cancer patients compared to healthy controls ($P < 0.01$) were detected with the BMW application. Peaks below 2 000 Da were discarded, as they result mainly from the SPA matrix. In either sample set, m/z values of $3.2*10^3$, $3.3*10^3$, 6.4×10^3, 6.6×10^3, 6.8×10^3, and 28×10^3 were differentially expressed. Expressions of m/z 2.7×10^3, 3.1×10^3, 4.2×10^3, 4.3×10^3, 4.5×10^3, 8.0×10^3, 8.9×10^3, 14×10^3, and 16×10^3 significantly differed only in sample set A.

With the BPS several classification trees were built. Tree characteristics of the best-performing trees with accompanying sensitivities and specificities are described in Table 1. Tree I and II were generated from sample set A, tree III and IV from sample set B, and tree V and VI from the combination of sample sets A and B. Tree sensitivities and specificities for trees I to IV were obtained using the second sample set as validation set. For trees V and VI, the sensitivity and specificity were calculated by randomly choosing one third of all data to be excluded from the tree building process for use as validation data afterwards. As shown in Table 1, m/z 3.3×10^3 was the most frequently observed classifier among these best trees. When removing this classifier from the tree-building model, equally- or better-performing trees were seen with m/z 28×10^3 as main classifier (trees II and VI). Other biomarkers used in the trees include m/z 3.1×10^3, 4.5×10^3, and 6.6×10^3. Of these, m/z 3.1×10^3 and 4.5×10^3 were more abundant in colorectal cancer serum samples compared to healthy controls, whereas the others were less abundant. Parts of representative MS-spectra for patients and controls are shown in Figure 2.

Biomarker selectivity

To determine the selectivity of the observed protein profiles for colorectal cancer when compared to other cancer forms, additional samples from patients with other tumours were analyzed. This third sample set was analyzed concomitantly with 17 of the previously analyzed samples from colorectal cancer patients (10 from sample set A, 7 from B) and 20 previously analyzed control samples (10 from either sample set) using the same assay procedures.

When examining peak intensity differences between cancer patients and healthy controls by means of the Biomarker Wizard application, most of the biomarkers for

Table 2 BMW expression differences of colorectal cancer biomarkers in other tumors

Group	3.1×10³ Da		3.3×10³ Da		4.5×10³ Da		6.6×10³ Da		28×10³ Da		5.9×10³ Da	
	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)
HC (n=20)	5.51 (2.4)		14.9 (4.73)		20.6 (9.16)		52.1 (7.4)		2.73 (1.25)		7.26 (4.23)	
CRC (n=17)	7.95 (4.64)	211	9.05 (3.81)	1.11	25.5 (5.99)	12.4	37.6 (9.17)	0.0388	1.44 (0.65)	0.801	10.84 (7.66)	161
BC (n=10)	8.87 (2.59)	1.13	11.51 (3.4)	103	19.44 (8.16)	538	42.7 (7.58)	5.59	1.44 (0.59)	3.69	4.51 (2.55)	43.0
NSCLC (n=8)	5.00 (2.93)	508	7.93 (3.38)	2.27	25.1 (7.93)	93.3	30.9 (11.2)	0.205	0.942 (0.45)	0.450	1.91 (2.13)	0.305
OC (n=10)	7.29 (3.77)	218	9.89 (2.52)	3.69	27.7 (8.67)	27.8	40.6 (8.65)	2.07	1.31 (0.45)	0. 968	2.98 (1.47)	1.32
PC (n=10)	7.65 (3.25)	131	9.59 (4.35)	7.21	29.6 (10.1)	18.4	36.5 (11.5)	0.967	1.26 (0.56)	1.35	2.73 (2.01)	1.86

For each m/z value, mean peak intensities (SD) are given with their P-values for the intensity difference with healthy controls (HC). BC: breast cancer; NSCLC: non-small cell lung cancer; OC: ovarian cancer; PC: prostate cancer.

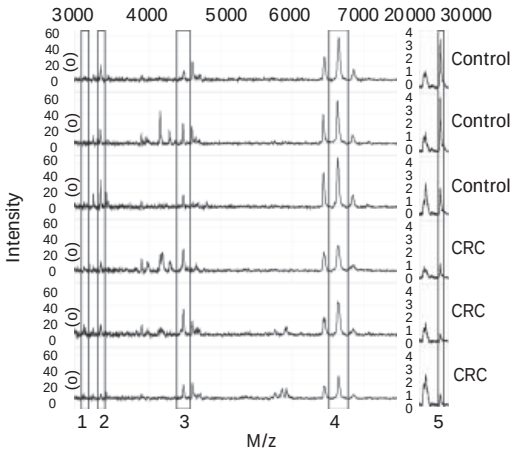


Figure 2 Spectra from colorectal cancer patients and controls. Biomarker proteins are boxed: 1=3.1×10³ Da, 2=3.3×10³ Da, 3=4.5×10³ Da, 4=6.6×10³ Da, 5=28×10³ Da.

colorectal cancer were found to be discriminative for other cancer forms as well (Table 2). Except for breast cancer samples, m/z 3.3×10³, 6.6×10³, and 28×10³ were markedly less abundant in all types of cancer compared to the control samples (P<0.01). For these tumour types, mean peak intensities were not significantly different from those for colorectal cancer, independent of patient characteristics (data not shown). There was a tendency for a significant increase of m/z 4.5×10³ in ovarian and prostate cancer (P<0.05). In breast cancer patients, no significance was reached for peak intensity differences of m/z 3.3×10³, and 4.5×10³ (P=0.10 and P=0.54, respectively). However, m/z 3.1×10³ was found to be significantly increased only in breast cancer samples (P=0.0011), but not in other cancer types.

A decision tree combining data from all tumour types was built with the BPS. Although most of the earlier observed biomarkers were discriminative for all other cancer forms as well, 76% of samples from colorectal cancer patients could be correctly distinguished from those of other cancers based on a classifier peak at m/z 5.9×10³. In samples from colorectal cancer, peak intensities for this m/z were slightly higher compared to the controls, whereas they were significantly lower than the controls in the other cancers (Table 2). In addition, data from the other tumour types were applied to the trees in Table 1. For all trees, more than 89% of patients with other cancers than colorectal cancer were classified as having colorectal cancer, except for tree VI, in which this was 78.4%.

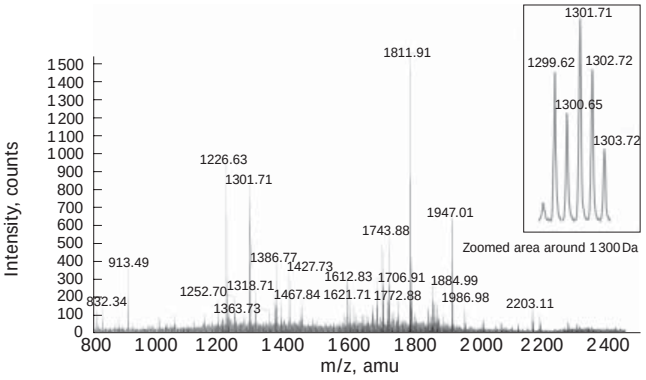
Biomarker purification and identification

Fractionation of whole serum from colorectal cancer patients and controls resulted in elution of the 6.6×10³-Da marker mainly in the flowthrough (pH 9), and the 28*10³-Da marker in the pH 4 fraction. Following concentration on YM-50 filters, the 6.6×10³-Da marker was seen in the 200 mL/L- and 300 mL/L-ACN eluates mostly, more purified from surrounding masses in the latter. The 28×10³-Da marker was present in the filter wash (1 mL/L TFA).

SDS-PAGE of selected eluates was performed on a 120 g/L Bis-Tris gel for the 28×10³-Da marker and a 180 g/L Tris-glycine gel for the 6.6×10³-Da marker. For this marker, both the 200 mL/L- and 300 mL/L-ACN eluates were placed on gel. A clear band was seen for the 28×10³-Da protein, which was divided in half for both passive elution and in-gel digestion. For the 6.6×10³-Da marker, a number of faint bands was seen in the 6000-Da region, in both the 200 mL/L- and 300 mL/L-ACN eluates. All were excised. Bands from the 200 mL/L-ACN eluate were subjected to passive elution, and from the 300 mL/L-eluate to in-gel digestion. Profiling of gel eluates on NP20 confirmed the masses to be indeed 6.6×10³ and 28×10³ Da.

Peptide mapping results revealed the identity of the 28×10³-Da marker to be apolipoprotein A-I. The theoretical mass of this protein is 28 078.62 Da in the SwissProt database and its pI=5.27. The apolipoprotein A-I identity was confirmed by tandem MS of the 1 299.62-, 1301.71-, 1612.83- and 1386.77-Da peptides in the tryptic digest (Figure 3).

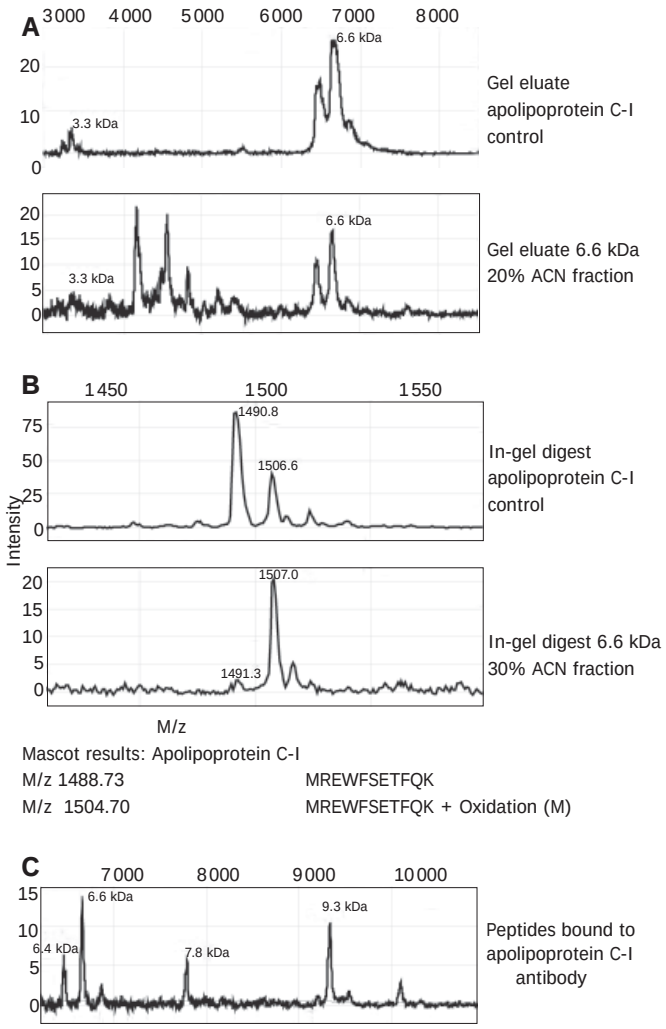
The 6.6×10³-Da marker was identified as apolipoprotein C-I, with a theoretical mass of 6 630.58 Da and pI=7.93. Spectra of this identification are shown in Figure 4. Confirmation of the apolipoprotein C-I identity was performed on protein A beads using a goat apolipoprotein C-I polyclonal antibody. The eluate's MS-spectrum clearly showed a large peak at 6.6×10³ and another prominent peak at 9.3×10³. The mass of the latter peak corresponded to that of apolipoprotein C-I precursor. In addition, the passive elution of the apolipoprotein C-I control (Figure 4A) showed a peak at 3.3×10³. The 3.3×10³-Da biomarker found in our sample sets A and B, as well as the set combined with other tumour types, consistently showed a high correlation with the 6.6×10³-Da one: the ratio of their



Mascot peptide mapping results: apolipoprotein A-I

Start-End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
70-83	1612.83	1611.82	1611.78	0.04	0	**LLDNWDSVTSTFSK**
84-101	2203.11	2201.10	2201.11	-0.01	1	LREQLGPVTQEFWDNLEK
131-140	1427.73	1426.72	1426.66	0.07	1	KWQEEMELYR Oxidation (M)
132-140	1299.62	1298.61	1298.56	0.05	0	**WQEEMELYR Oxidation (M)**
143-155	1467.84	1466.83	1466.78	0.05	1	VEPLRAELQEGAR
251-262	1386.77	1385.76	1385.71	0.05	0	**VSFLSALEEYTK**
185-195	1301.71	1300.70	1300.64	0.06	0	**THLAPYSDELR**

Figure 3 Peptide mapping of 28×10^3-Da apolipoprotein A-I. MS spectrum of the 28×10^3-Da in-gel tryptic digest. Results from the MASCOT search for protein identification include start and end positions of the found peptide sequence starting from the amino acid terminal of the whole protein, the observed m/z, transformed to its experimental mass [Mr(expt)], the calculated mass [Mr(calc)] from the matched peptide sequence, as well as their mass difference (Delta), the number of missed cleavage sites for trypsin (Miss) and the peptide sequence. Peptides in bold were sequenced with tandem MS using Q-TOF for confirmation.



Mascot results: Apolipoprotein C-I

M/z 1488.73	MREWFSETFQK
M/z 1504.70	MREWFSETFQK + Oxidation (M)

Figure 4 Identification of apolipoprotein C-I. **A:** Parts of the MS spectra of the gel eluates of an apolipoprotein C-I control and the 6.6×10^3-Da protein isolated from HC serum run on the same gel. **B:** Parts of the MS spectra of the in-gel digests of an apolipoprotein C-I control and the 6.6×10^3-Da protein isolated from HC serum and the results of sequencing of these two peptides with tandem MS. **C:** Part of the MS spectrum of the eluate from the apolipoprotein C-I antibody. Apart from the expected peaks at 9.3×10^3, apolipoprotein C-I precursor, and 6.6×10^3, a 6.4×10^3-Da peak is seen, which is a known fragment of apolipoprotein C-I missing two N-terminal amino acids. The mass at 7.8×10^3 is unknown and does not correspond to any of the apolipoproteins with which antibody cross-reactivity can occur. It is possibly an intermediate splice form of the precursor protein.

peak intensities was quite constantly ranging between 3.5 and 4.0 (Table 2). This supports the fact that the observed 3.3×10^3-Da marker is actually a doubly charged artefact of the 6.6×10^3-Da protein. The 3.1×10^3-Da protein was lost during the purification process and was therefore directly sequenced on-chip. It was identified as a 27-amino acid N-terminal fragment of albumin with sequence: DAHKS EVAHRFKDLGEENFKALVLIAF. The identity of the 4.5×10^3-Da protein is still under investigation.

Serum CRP, TRF and CEA levels

Evaluation of the extent of a possible acute phase reaction was done by measurement of CRP and TRF levels. Mean TRF levels in the patient and control group were 2.37 g/L (range, 1.20-3.60 g/L) and 2.59 g/L (range, 1.90-4.00 g/L), respectively (P=0.037, non-parametric Mann-Whitney U test). Mean CRP levels in either group were 29.0 mg/L (range, 0 - 213 mg/L) and 3.70 mg/L (range, 0 - 29.2 mg/L) (P<0.000, non-parametric Mann-Whitney U test). Although there was a significant difference in the levels of these acute phase reactants in the patients and controls, the mere presence of an acute phase response was not a good predictor for colorectal cancer: the sensitivities of CRP and TRF were 51.9% (40/77) and 22.1% (17/77), respectively, using the clinical cut-off values [specificities 88.8% (71/80) and 96.3% (77/80), respectively]. In addition, CRP and TRF levels were included in the BMW data files for the tree-building process with the BPS, in order to evaluate their capability to distinguish colorectal cancer patients and healthy controls. Neither CRP, nor TRF concentrations were as good a classifier as the m/z values in the generated trees.

Mean serum CEA in the colon group was significantly higher (mean 326.2 μg/L, range <1-9 452) compared to the control group (mean 2.23 μg/L, range <1-18.98) (P<0.001, non-parametric Mann-Whitney U test). Using a cut-off value of 5 μg/L, its sensitivity and specificity were found to be 75.3% and 95.0%, respectively, for all samples combined. This sensitivity was lower than that for the proteins in the classification trees generated with all samples (V and VI), but the specificity of CEA in this population was higher. Assessing CEA sensitivity in the total sample set according to colorectal cancer stage, using the 5 μg/L cut-off, resulted in correct classification of 0 of 1 Dukes' A, 0 of 2 Dukes' B, 3 of 12 (25.0%) Dukes' C, and 51 of 57 (89.5%) Dukes' D. In comparison, using the total sample set and the trees generated with the sets (V and VI), 1 of 1 Dukes' A, 1 of 2 Dukes' B, 11 of 12 (91.7%)

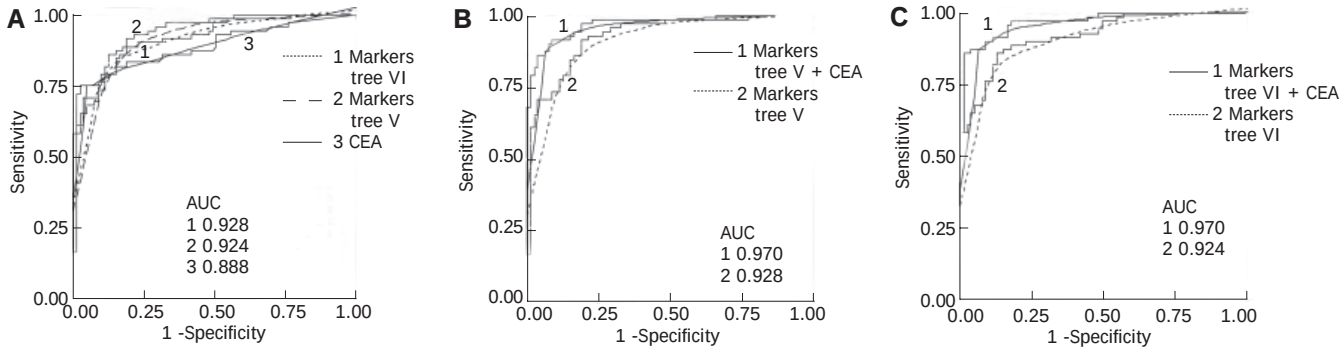


Figure 5 ROC curves for biomarker proteins from trees V and VI with and without log(CEA). Areas under the curve (AUC) are given for each model.

Dukes' C, and 47 of 57 (82.5%) Dukes' D were correctly classified. In addition, logistic regression was performed for CEA and the markers from tree V and VI, and receiver operating characteristic (ROC) curves were generated. As shown in Figure 5, combining the markers from each tree with log-transformed CEA values yielded a higher area under the ROC curve than for either alone.

DISCUSSION

In this study, five biomarker proteins were detected that were able to reliably distinguish colorectal cancer patients and healthy controls using the SELDI-TOF MS technique for protein profiling. Two of these were identified as apolipoprotein C-I (6.6×10^3 Da) and apolipoprotein A-I (28×10^3 Da). Using the ProteinChip Software, the 3.3×10^3-Da protein could be identified as a doubly charged form of the 6.6×10^3-Da apolipoprotein C-I, which was confirmed by its appearance in the MS spectrum of pure apolipoprotein C-I isolated from a gel. The m/z of 3.1×10^3 was found to be an N-terminal fragment of albumin. In addition, the detection of these biomarkers' expression difference was shown to be reproducible on two separate occasions, considering the obtained classification tree sensitivity and specificity between 65% and 90% when using the second sample set as a blinded validation set. Such reproducible detection is imperative for any future use as a clinical tool. Sensitivities and specificities obtained with data from the blinded sample set were comparable to those obtained by cross-validation within one sample set (data not shown), which supports the fact that there was no additional misclassification of samples due to experimental variability between the two sample sets.

Several reports have been made of differential expression of the same m/z values in colorectal cancer, even though different chip surfaces were used. Yu *et al*[17] reported a 3 329- and 6 669-Da protein to be differentially expressed on a hydrophobic chip surface, that were not selected in the final diagnostic pattern. In the same study, a 4 477-Da protein, which was a classifier in the final pattern, was also up-regulated in colorectal cancer patients[17,18]. The 6.6×10^3- and 3.3×10^3-Da proteins were detected by Yu *et al*[17] but not identified. It is likely that these are apolipoprotein C-I and its doubly charged form, since this is a very hydrophobic protein and retention on a hydrophobic chip surface is very plausible. In fact, in our study population, these m/z values were also seen on hydrophobic chips (data not shown). This appearance of (likely) the same colorectal cancer biomarkers in different laboratories underlines their validity. Also, a 5.9×10^3-Da protein was reported, which was an up-regulated biomarker in serum of colorectal cancer patients[17,18]. Despite lack of significance in our study, there was a tendency for our 5.9×10^3-Da protein to be higher in patients than controls, which is consistent with the result from Yu *et al*[17] and indicates this may be the same protein. Data from a proteomic study by our group[19] on breast cancer patients have shown a 5.9×10^3-Da down-regulated protein in this cancer type, which is in concordance with the 5.9×10^3-Da protein in the current study. The protein in the former study was identified as a fragment of fibrinogen alphaE chain.

Apolipoprotein C-I is primarily synthesised in the liver and, to a minor degree, in the small intestine. Its function resides mainly in lipoprotein metabolism[20]. It is originally formed as a pro-peptide of 9.3×10^3 Da, which generates the mature protein upon cleavage during translation. To our knowledge, no previous reports about apolipoprotein C-I down-regulation in cancer have been made as yet. However, a 6.6×10^3-Da protein was detected and identified as apolipoprotein C-I in another SELDI-TOF MS study, being decreased in hemorrhagic *versus* ischemic stroke and hemorrhagic stroke *versus* controls on a strong anion exchange surface at pH 9[21]. Apolipoprotein A-I is synthesised both in the liver and small intestine and is a major constituent of HDL apolipoprotein. It is a known negative acute phase reactant, of which decreased expression has been described in several cancers, including a SELDI-TOF MS study on ovarian cancer[22-25]. In the latter study an immunoassay was peformed. In contrast to our data, the authors found no decreased apolipoprotein A-I levels in colorectal cancer. However, apolipoprotein A-I levels assessed by immunoassay may reflect concentrations of both bound pro-apolipoprotein A-I and apolipoprotein A-I. Increased expression of apolipoprotein A-I has been described in tissue of both liver metastases and, to a lesser extent, primary tumours of colorectal origin[26]. The observed decrease in serum levels in our study thus may be due to decreased liver synthesis. Other human proteomics studies in which differential expression

of apolipoprotein A-I has been described include a SELDI-TOF MS analysis of plasma from patients with diabetes, and several studies using 2D-gel electrophoresis in old *versus* young brain tissue, cerebrospinal fluid of patients with Alzheimer's disease, serum during infection with hepatitis B virus, and plasma during acute coronary syndrome[27-31]. In all these diseases, decreased levels of apolipoprotein A-I were observed. To our knowledge, the albumin fragment that was found in this study has not been described in the literature before. Albumin is synthesised with an 18-amino acid signal peptide and a 6-amino acid pro-peptide. Over-expression of this specific fragment may be caused by enhanced proteolytic activity, as increased proteolysis is common in cancer invasion and metastasis[32]. However, for this fragment, a correlation with sample age was seen in the colorectal cancer group (data not shown). Thus, it cannot be ruled out that it is a product of protein degradation upon storage.

Although the identification of apolipoprotein C-I and apolipoprotein A-I as biomarkers suggests an acute phase response, comparison with routine markers for establishing such a response, CRP and TRF, shows that our biomarkers are much more sensitive for colorectal cancer than these. The value of our biomarkers for detection of colorectal cancer was also evaluated by comparison with the predictive value of CEA. Sensitivity of our biomarkers was higher than that of CEA considering all samples. Stratification by Dukes' stages showed a significant better sensitivity of our classification trees (91.7%, 11/12) compared to CEA (25.0%, 3/12) in Dukes' C colorectal cancer, although at stage D CEA performed better. Combining log-transformed CEA in a logistic regression model with the markers in the trees resulted in a higher AUC in the ROC curve than for either log(CEA) or the combined tree classifiers alone. This indicates that our markers provide additional information to CEA values. CEA sensitivity has been reported to be lower in earlier stages of colorectal cancer. Sensitivity has been reported to vary between 3% and 66.7% for Dukes' A to D staged disease[2,18,33]. No conclusions can be drawn on the performance of our classification trees at earlier stages of colorectal cancer due to limited samples, but 2 of 3 patient samples from stage A and B were correctly classified by the trees and none when using the clinical cut-off for CEA.

Our results showed that it is very important to compare any biomarkers found for a certain type of cancer with those for other tumour types. This is lacking in most of the SELDI-TOF MS studies published so far. We found that most of our biomarker proteins were differentially expressed in other cancers as well. Lack of a significant difference at m/z 3.3×10^3 and 4.5×10^3 in breast cancer patients could be explained by the large proportion of early disease (9 of 10 stage 2) in this group compared to the others (mainly stage 3 and 4). However, since m/z 3.3×10^3 is the doubly charged 6.6×10^3-Da protein, which was significantly less expressed in breast cancer, this lack of significance is more likely due to slight differences in ionisation of this protein in this group. The fact that the 3.1×10^3-Da protein is not significantly different in ovarian and prostate cancer may result from the limited sample size, as for the smaller colorectal cancer group in this analysis with comparable mean peak intensity no significant difference at m/z 3.1×10^3 was observed either, although in sample sets A and B it was.

Even though most of our biomarkers are not specific for colorectal cancer, a potential role for them lies in therapy evaluation, disease surveillance or prognosis, possibly combined with CEA or other available markers. At present, CEA is recommended for monitoring chemotherapy, but no studies showing any benefit on survival, quality of life, or reduction of costs are available, although serial CEA testing may lead to earlier detection of progressive disease[5]. In addition, specificity of CEA for treatment monitoring can be compromised by transient increases during treatment with various chemotherapeutic drugs, such as 5-flourouracil and levamisole[5]. Since the expression profiles of our reported markers reliably reflect presence of cancer, be it colorectal cancer or not, changes in expression levels may correspond to response to therapy or disease progression and provide additional information to CEA levels.

In conclusion, our results show that SELDI-TOF MS is a suitable technique to find new serum biomarkers for colorectal cancer. The markers we have found in this study reliably distinguish colorectal cancer patients from healthy persons. Although not specific for colorectal cancer, they have a potential role as markers in treatment monitoring, disease surveillance, or prognosis, possibly in combination with other available markers. To extend the study population and evaluate the ability of our biomarkers to detect early-stage tumours and polyps, a prospective study is currently ongoing.

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Psychological impact of chronic hepatitis C: Comparison with other stressful life events and chronic diseases

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Abstract

AIM: To examine the psychological impact of chronic hepatitis C (CHC) diagnosis in a large cohort of CHC patients as compared with other stressful life events and chronic diseases carrying a risk of life-threatening complications.

METHODS: One hundred and eighty-five outpatients with compensated CHC were asked to self-grade, using a 100-mm visual analogue scale (VAS), the degree of stress caused by the learning of CHC diagnosis and the perceived severity of their disease. Diagnosis-related stress was compared to four other stressful life events and perceived CHC severity was compared to four other common chronic diseases.

RESULTS: Learning of CHC diagnosis was considered a major stressful event (mean ± SD scores: 72±25), significantly less than death of a loved-one (89±13, $P < 0.0001$) and divorce (78 ± 23, $P < 0.007$), but more than job dismissal (68 ± 30, $P < 0.04$) and home removal (26±24, $P < 0.0001$). CHC was considered a severe disease (74±19), after AIDS (94±08, $P < 0.001$) and cancer (91±11, $P < 0.001$), but before diabetes (66±23, $P < 0.001$) and hypertension (62±20, $P < 0.001$). Perceived CHC severity was not related to the actual severity of liver disease, assessed according to Metavir fibrosis score. In multivariate analysis, diagnosis-related stress was related to perceived disease severity ($P < 0.001$), trait anxiety ($P < 0.001$) and infection through blood transfusion ($P < 0.001$).

CONCLUSION: Our results show the considerable psychological and emotional burden that a diagnosis of CHC represents, even in the absence of significant liver disease. They should be taken into account when announcing a diagnosis of CHC in order to reduce its negative effects.

Key words: Hepatitis C; Stressful life event; Perceived severity; Visual analogue scale

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INTRODUCTION

Despite the fact that chronic hepatitis C (CHC) may lead to life-threatening complications such as cirrhosis, liver failure and hepatocellular carcinoma, most patients with CHC are asymptomatic and unaware of their liver disease prior to diagnosis. Nonetheless, these patients consistently report a significant reduction in health-related quality of life (HRQOL) as compared with population controls[1-6]. This impairment of HRQOL has been documented even in the absence of significant liver disease[3] and is usually improved after sustained viral clearance[4,5]. In addition, clinically significant emotional distress and depressive disorders have been reported in untreated CHC patients with a prevalence ranging from 11 to 53%[2,7-15], questioning the role of HCV itself in their occurrence[16]. Whether these disorders are due to the uncertainty of living with a chronic disease with potential life-threatening complications or to other psychosocial factors remains unclear. However, it has been recently shown that CHC patients aware of their diagnosis had worse HRQOL scores as compared with unaware seropositive patients[6,17], suggesting that the psychological impact of diagnosis knowledge may play an important role.

The aim of this prospective study was to examine the psychological impact of CHC diagnosis in a large cohort of CHC patients as compared with other stressful life events and chronic diseases carrying a risk of life-threatening complications.

MATERIALS AND METHODS

Study population

A total of 185 patients with a confirmed diagnosis of HCV infection (defined as detectable hepatitis C antibodies and HCV RNA in serum) attending the Hepatology Clinics at the University of Bordeaux were studied. Exclusion criteria were: age below 18 years, decompensated cirrhosis, co-infection by hepatitis B virus or human deficiency virus (HIV), current psychotic or manic disorders, obvious intellectual impairment, and inability to communicate in French.

Measures

Immediately prior to or following an outpatient clinic appointment, and after their written informed consent had been obtained, participants were asked to self-grade, using a 100-mm visual analogue scale (VAS), the degree of stress caused by the learning of CHC diagnosis. On that scale, the left endpoint 0 was defined as not stressful at all, the right endpoint 100 as extremely stressful. There were no further marks on the line. Instructions were as follows: "Put a mark on the line at the point that best describes how stressful you consider the learning of hepatitis C diagnosis". The degree of stress experienced was indicated by the distance in millimeters from the left endpoint. Perceived disease severity was assessed using the same method. VAS has been shown to be a reliable tool to assess subjective variables, such as stress[18], pain[19] and fatigue[20].

In addition to validate the reproducibility of measurements over time, a second assessment was performed 3 mo later in a subgroup of randomly selected patients.

Trait anxiety was assessed using the Spielberger State Trait Anxiety Inventory[21], a 20-item standardized self-report scale ranging from 20 (low tendency to be anxious) to 80 (high tendency to be anxious).

The influence on diagnosis-related stress of age, gender, marital status, educational level, route of HCV transmission, time since diagnosis, antiviral therapy, perceived severity of illness, actual severity of liver disease (assessed according to the fibrosis score of the METAVIR scoring system[22] in patients in whom a liver biopsy specimen was available), and trait anxiety was also studied.

Comparison with other stressful life events and chronic diseases

Using VAS mean scores, diagnosis-related stress was compared to four other stressful life events (i.e., home removal, job dismissal, divorce, or death of a loved-one) selected from the Paykel life-event scale[23]. Perceived CHC severity was compared to four other common chronic diseases carrying a risk of life-threatening complications (i.e., hypertension, diabetes mellitus, cancer and AIDS).

Statistical analysis

VAS scores for diagnosis-related stress and perceived disease severity were expressed as mean ± SD. Chi-square test was used for categorical variables and one-way analysis of variance (ANOVA) for continuous variables. A P value < 0.05 was considered statistically significant. Using paired Student's t-test, test-retest reliability was assessed for VAS mean scores in a randomly selected sample of patients. For the study of factors associated with diagnosis-related stress, ANOVA was used for both categorical (gender, educational level, marital status, route of transmission, antiviral therapy, and histological severity of liver disease) and continuous variables (age, perceived disease severity and trait anxiety). For the later, patients were divided into two groups according to the median value. Multivariate analysis was performed including variables significantly associated with diagnosis-related stress ($P < 0.10$).

Table 1 Characteristics of the 185 patients studied

	n = 185 (%)
Age (yr)	45±11
Gender (male/female)	111/74
Educational level	
No diploma	24 (13)
High school	83 (45)
College undergraduate	35 (19)
College graduate	43 (23)
Marital status	
Single/divorced	57 (31)
Married	92 (50)
Non-married couple	36 (19)
Routes of transmission for HCV	
Blood transfusion	74 (40)
Intravenous drug use	48 (26)
Others*	63 (34)
Histological severity (Metavir fibrosis score)	n=169
F0-1	39 (23)
F2	83 (49)
F3	19 (11)
F4	28 (17)
Trait anxiety (STAI)	45±11 (20-80)

RESULTS

Study population

The characteristics of the 185 patients are shown in Table 1. There were 111 men and 74 women with a mean age of 45±11 years. The study group had a broad distribution of educational background. Seventy-four (40%) patients were infected through blood transfusion, whereas 48 (26%) were infected through intravenous drug use. The mean time since CHC diagnosis was 2.9±2.6 years. Fifty-nine (32%) patients were receiving antiviral therapy at the time of the study. Of the 169 patients with available liver biopsy data, 28 (17%) had evidence of cirrhosis. The mean STAI-Y score for trait anxiety was 45±11 (range 22-80). This score did not differ from measures obtained in a community sample [24].

VAS scores for diagnosis-related stress and perceived disease severity

Mean±SD scores for diagnosis-related stress were 72±25 (range: 5-100). Mean±SD scores for perceived disease severity were 74±19 (range: 15-100). Three months later, the second assessment in a subgroup of 30 randomly selected patients showed that all VAS scores were reproducible over time (no difference was observed for

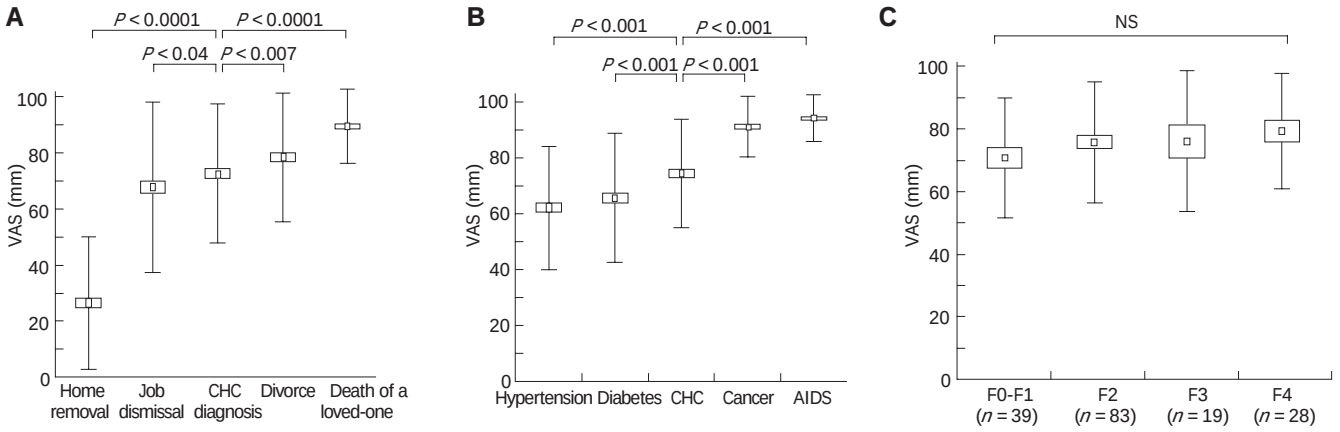

Figure 1 (**A**) Comparison between mean visual analogue scale (VAS) scores for chronic hepatitis C (CHC) diagnosis-related stress and four other stressful life events (i.e., home removal, job dismissal, divorce, and death of a loved-one) selected from the Paykel life-event scale[23] in the 185 patients. *P* values are for post-hoc comparisons made after significant one-way ANOVA ($F = 249.32$, $P < 0.0001$). (**B**) Comparison between mean visual analogue scale (VAS) scores for perceived chronic hepatitis (CHC) severity and four other common chronic diseases carrying a risk of life-threatening complications (i.e., hypertension, diabetes, cancer and AIDS) in the subgroup of 185 patients. *P* values are for post-hoc comparisons made after significant one-way ANOVA ($F = 173.81$, $P < 0.001$). (**C**) Mean visual analogue scale (VAS) scores for perceived chronic hepatitis (CHC) severity according to actual severity of liver disease assessed using METAVIR fibrosis score (F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis and few septa; F3: numerous septa without cirrhosis; F4: cirrhosis) in the 169 patients with available liver specimen. In Figures A, B and C, the upper and lower limits of the boxes and the middle square indicate the standard error and the mean, respectively. The upper and lower horizontal bars indicate the standard deviation.

any item, using a paired *t* test).

Comparison with other stressful life events and chronic diseases

Mean±SD scores for stress were as follows: death of a loved-one (89±13), divorce (78±23), CHC diagnosis (72±25), job dismissal (68±30) and home removal (26±24) (Figure 1A). CHC diagnosis was significantly more stressful than job dismissal ($P < 0.04$) and home removal ($P < 0.0001$) but less than divorce ($P < 0.007$) and death of a loved-one ($P < 0.0001$).

Mean VAS scores for perceived disease severity were as follows: AIDS (94±08), cancer (91±11), CHC (74±19), diabetes mellitus (66±23) and hypertension (62±20) (Figure 1B). CHC was considered significantly more severe than diabetes mellitus ($P < 0.0001$) and hypertension ($P < 0.0001$) but less than cancer ($P < 0.001$) and AIDS ($P < 0.001$).

Relationship between perceived disease severity and actual severity

Mean VAS scores for perceived disease severity did not differ according to the Metavir fibrosis stages in the 169 patients in whom a liver specimen was available (Figure 1C).

Factors associated with diagnosis-related stress

In univariate analysis, diagnosis-related stress was associated with female sex ($P < 0.003$), infection through blood transfusion ($P < 0.002$), high perceived disease severity ($P < 0.001$) and high trait anxiety ($P < 0.001$) (Table 2). Stress tended to be higher in patients with low education level, although the difference did not reach statistical significance ($P = 0.07$). Scheffé post hoc tests revealed that stress scores were significantly higher in

Table 2 Factors associated with diagnosis-related stress in univariate analysis in the 185 patients

	Diagnosis-related stress (mean VAS scores)	*P*
Age (yrs)		
<46	73±25	
⩾46	72±24	NS
Gender		
Male	66±26	
Female	79±22	<0.003
Educational level		
< High school	75±24	
⩾ High school	69±25	0.07
Marital status		
Single/divorced	73±25	
In couple	72±24	NS
Routes of transmission for HCV		
Blood transfusion	80±20	
Intravenous drug use	65±27	
Others	68±26	<0.002
Antiviral therapy		
Ongoing	69±25	
Untreated	74±25	NS
Histological severity of liver disease ($n = 169$)		
F0-F1-F2 ($n = 122$)	74±23	
F3-F4 ($n = 47$)	70±29	NS
Time since diagnosis (yrs)		
<2	71±26	
⩾2	73±24	NS
Perceived disease severity (VAS)		
<75	59±23	
⩾75	83±21	<0.001
Trait anxiety (STAI)		
<45	64±27	
⩾45	81±19	<0.001

For continuous variables (age, time since diagnosis, perceived disease severity and trait anxiety) patients were divided into two groups according to the median value.

Table 3 Factors associated with diagnosis-related stress in multivariate analysis in the 185 patients

	Diagnosis-related stress (100mm VAS score)[2]	
	β^1	P
Gender : (male: 1, female: 2)	0.02	0.76
Routes of transmission: (drug user = 1; other = 2, blood transfusion = 3)	0.17	< 0.01
Educational level: (< high school:1, ⩾ high school:2)	-0.03	0.58
Anxiety (STAI-Y score)	0.30	< 0.001
Perceived disease severity (100mm VAS score)	0.43	< 0.001

[1]regression coefficient.
[2]Regression model: R = 0.60, R^2 = 0.36, adjusted R^2 = 0.35, F(5, 171) = 19.65, P < 0.001.

patients infected through blood transfusion than in those infected through intravenous drug use (P < 0.001). No difference was observed for patients infected through other routes of transmission. Stress did not differ between untreated and treated patients. In the latter patients, stress did not differ according to virological response.

In multivariate analysis, stress was related only to perceived disease severity (P < 0.001), trait anxiety (P < 0.001) and infection through blood transfusion (P < 0.01). These parameters accounted for 36% of the variance of diagnosis-related stress (R^2 = 0.36; P < 0.001; Table 3).

DISCUSSION

Our results show that learning one has contracted HCV infection, with its implications for future health and behaviour, is a major stressful event (with mean scores of 72 ± 25 on a 100-mm visual analogue scale). By comparison with other stressful life events selected from the Paykel life-event scale[23], ranging from minor (i.e., home removal) to major (i.e., death of a loved one), CHC diagnosis was considered less stressful than death of a loved one (89 ± 13, P < 0.0001) and divorce (78 ± 23, P < 0.007), but more than job dismissal (68 ± 30, P < 0.04) and home removal (26 ± 24, P < 0.0001). These results emphasize the considerable emotional and psychological burden that CHC diagnosis represents in patients' life. So far, the psychological issues that CHC patients face remain poorly understood. In the studies having reported clinically significant emotional distress and depressive disorders in patients with CHC[2,13-15], the psychological impact of diagnosis knowledge was not taken into account. However, it is important to distinguish between psychological reactions to the knowledge that one has been infected with HCV and the direct effects of the virus itself as evidenced by the results of the present study. Our findings are consistent with those of Rodger *et al*[6] who compared 15 patients who were aware of having HCV infection with 19 subjects who were unaware of having HCV infection, and found that the former had significantly poorer HRQOL scores. More recently, Dalgard *et al*[17] have confirmed these results in a cohort of 199 Swedish active drug user patients, showing that those who believed they were infected had a lower HRQOL scores than those who believed they were not infected. Taken together, these results provide evidence of the considerable negative impact of the knowledge of the diagnosis of CHC on patients' perception of their psychological well-being, known for other chronic diseases as the "labeling" effect[25]. Such an effect has recently been illustrated in a study by Zickmund *et al*[26], showing that stigmatization was common in patients with CHC and was associated with a lower quality of life and with a deterioration of social support.

There are increasing evidences that CHC infection may be associated with significant cerebral dysfunction. Several authors have reported cognitive impairment and central nervous system functional abnormalities in CHC patients compared with uninfected controls, using P300 event-related potentials, a sensitive electrophysiologist test of cognitive processing[27] or magnetic resonance spectroscopy[28-31]. Such findings suggest a direct biological effect of HCV infection on cerebral function. Whether cognitive dysfunction is an important determinant of impaired HRQOL in CHC remains to be determined.

As natural history of chronic HCV infection remains poorly defined[32], it is not surprising that CHC diagnosis, a disease with uncertain outcome, raises significant concerns about future health status. Indeed, CHC was perceived as a severe disease (74 ± 19), after AIDS (94 ± 08, P < 0.001) and cancer (91 ± 11, P < 0.001), but before diabetes (66 ± 23, P < 0.001) and hypertension (62 ± 20, P < 0.001). Interestingly, perceived CHC severity was not related to the actual severity of the disease, as assessed by the severity of liver histological lesions. This finding is consistent with the results of Foster *et al*[3] who showed that the significant impairment of HRQOL experienced by CHC patients could not be attributed to the histological severity of liver disease. It is also in keeping with studies[33-35] conducted in patients with hypertension or chronic respiratory diseases, showing that perception of a disease usually correlates poorly with its medically defined characteristics. In addition, the patients' perception of CHC severity seemed to have a more significant impact on their psychological status than did the histological severity of their liver disease as evidenced by multivariate analysis, showing that diagnosis-related stress was mainly related to perceived CHC severity, trait anxiety and infection through blood transfusion. An explanation for that latter finding may be the fact that patients infected through blood transfusion had no reason to expect such a health problem. By contrast, former drug users likely knew, given their past lifestyle, they were at risk of contracting severe diseases, such as HIV/AIDS, hepatitis B or C, with related-consequences for their future health.

Although our patient population does not represent a random sampling of CHC patients, we believe that it is representative of the general population of CHC patients. The demographic and clinical characteristics of our study population, including routes of transmission and proportion of patients with cirrhosis at the time of diagnosis, are similar to that reported in large surveys of patients with CHC in France[36,37]. In addition, all

VAS scores were reproducible over time in a sample of randomly selected patients. Although some of our patients received antiviral therapy (32%), diagnosis-related stress and perceived disease severity mean VAS scores did not differ between treated and untreated patients.

Finally, our findings may have important implications for clinicians who take care of patients with CHC. They highlight the importance of communication between patients and their physician and the crucial role of the specialist in providing adequate and accessible information for patients to cope with their condition[38].

In conclusion, our results show the considerable psychological and emotional burden that a diagnosis of CHC represents, even in the absence of significant liver disease. They should be taken into account when announcing a diagnosis of CHC in order to reduce its negative effects.

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Efficient expression of histidine-tagged large hepatitis delta antigen in baculovirus-transduced baby hamster kidney cells

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Abstract

AIM: To study the baculovirus/mammalian cell system for efficient expression of functional large hepatitis delta antigen (L-HDAg).

METHODS: A recombinant baculovirus expressing histidine-tagged L-HDAg (L-HDAgH) was constructed to transduce baby hamster kidney (BHK) cells by a simplified transduction protocol.

RESULTS: The recombinant baculovirus transduced BHK cells with efficiencies higher than 90% as determined by flow cytometry. The expression level was significantly higher than that obtained by plasmid transfection and was further enhanced 3-fold to around 19 pg/cell by the addition of 10 mmol/L sodium butyrate. Importantly, the expressed L-HDAgH was localized to the cell nucleus and correctly isoprenylated as determined by immunofluorescence labeling and confocal microscopy. Moreover, L-HDAgH interacted with hepatitis B surface antigen to form virus-like particles.

CONCLUSION: The fusion with histidine tags as well as overexpression of L-HDAgH in the baculovirus-transduced BHK cells does not impair the biological functions. Taken together, the baculovirus/mammalian cell system offers an attractive alternative for high level expression of L-HDAgH or other proteins that require extensive post-translational modifications.

Keywords: Baculovirus; Hepatitis delta virus; L-HDAg; Mammalian cell; Protein expression; Transduction

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INTRODUCTION

Hepatitis delta virus (HDV) is a subviral satellite and requires hepatitis B virus to supply envelope proteins (HBsAg) for its packaging, secretion and infection[1, 2]. HDV superinfection of HBV carriers can lead to fulminant hepatic failure and very likely the progression to liver cirrhosis[3], consequently HDV imposes a huge threat to the health of 350 million HBV carriers worldwide. However, effective vaccines or diagnostic assays remain unavailable. The RNA genome of HDV encodes the large and small forms of hepatitis delta antigens (L-HDAg and S-HDAg). The 24 kd S-HDAg and 27 kd L-HDAg are identical in sequence except that L-HDAg contains additional 19 amino acids at the C-terminus[1, 4]. The C-terminus extension of L-HDAg allows for the isoprenylation which is crucial for the interaction with S-HDAg and HBsAg for the formation of virions. It has been established that cell-mediated immunities are important in controlling HDV infection[5, 6], thus an effective T-cell stimulating HDV vaccine would be highly desired. In this regard, L-HDAg may represent an excellent vaccine candidate because L-HDAg (expressed by a DNA vaccine) induces significant titers of anti-HDV antibodies as well as strong cell-mediated immune responses in mice[7]. Moreover, L-HDAg is a dominant repressor for HDV replication[8], thus implicating its application as an anti-HDV drug in addition to being a vaccine. However, efforts to characterize and evaluate the immunological properties of L-HDAg have been hampered due to the lack of a proper method for efficient expression and purification of L-HDAg.

Baculovirus (*Autographa californica* multiple nuclear polyhedrosis virus, AcMNPV) has been widely utilized for protein expression in insect cells. Since the finding that baculovirus is capable of transducing mammalian cells in 1995, baculovirus has been developed as a vector for *in vitro* and *in vivo* gene therapy studies and many other applications (for review, see[9, 10]). Recently, the applications

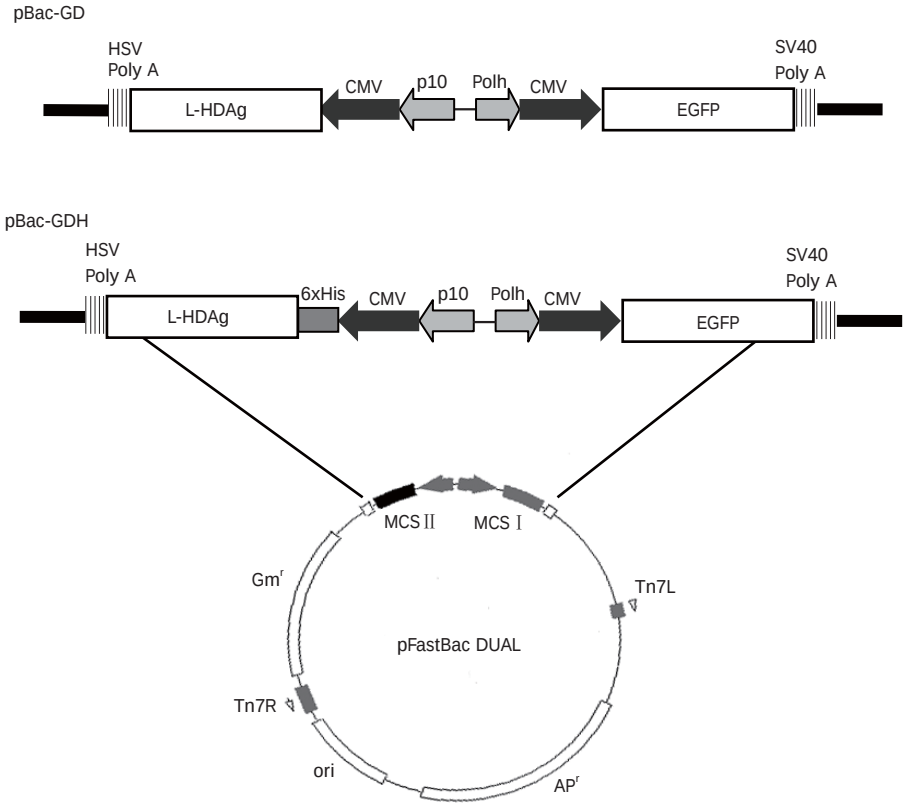


Figure 1 Schematic illustration of recombinant donor plasmids pBac-GDH and pBac-GD

of baculovirus have been further expanded to the identification of MHC class I mimotopes[11] and genetic modification of primary chondrocytes[12]. Furthermore, our group has also demonstrated the feasibility of producing HDV virus-like particles in Huh-7 cells[13]. All these studies suggest that baculovirus may be an ideal tool for the expression of functional, complex eukaryotic proteins owing to the following advantages: (1) Baculovirus transduction is highly efficient for many cell types and generally causes no appreciable cytopathic effect[10,14]; (2) The large AcMNPV genome (about 130 kb) confers the virus huge cloning capacity to harbor multiple genes or large inserts[15]; (3) The construction, manipulation and production of recombinant baculovirus are easy.

Considering the need for efficient expression of L-HDAg and the advantages of baculovirus/mammalian cell system, we constructed a recombinant baculovirus encoding histidine-tagged L-HDAg (L-HDAgH) for rapid and high level expression in transduced BHK cells. The high level expression and subsequent purification may allow us to obtain sufficient quantities of functional L-HDAgH for further evaluation of its immunological properties and its potential as a vaccine candidate. The success of this study supports the notion that the baculovirus/mammalian cell system can be a platform for efficient expression of eukaryotic proteins requiring post-translational modifications.

MATERIALS AND METHODS

Generation of recombinant baculoviruses

The transfer plasmid was generated using pFastBac™ DUAL (Invitrogen) as the backbone, which contained two multiple cloning sites (MCS). The gene encoding enhanced green fluorescent protein (EGFP), along with the upstream cytomegalovirus immediate-early (CMV-IE) promoter, was amplified by polymerase chain reaction (PCR) from pEGFP-C1 plasmid (Clontech) and inserted into MCS I under the polyhedrin promoter. The cDNA encoding L-HDAg was PCR-amplified from p2577-1L[16] and subcloned into pcDNA4/HisMax-TOPO (Invitrogen), thus a His_6 tag was fused at the N-terminus of L-HDAg under the control of CMV-IE promoter. The whole expression cassette was PCR-amplified again and subcloned into *Kpn* I site of MCS II. The resultant plasmid was designated pBac-GDH (Figure 1). Another plasmid, pBac-GD, was previously constructed to harbor the genes encoding L-HDAg and EGFP[13] (Figure 1). The recombinant baculoviruses were subsequently generated using the Bac-to-Bac® system (Invitrogen) and designated as Bac-GDH and Bac-GD. Another recombinant baculovirus co-expressing HBsAg and EGFP, Bac-GB, was constructed previously[13].

Cells and medium

BHK cells were cultured in Dulbecco's minimal essential medium (DMEM, Sigma) supplemented with 100 mL/L heat inactivated fetal bovine serum (FBS; Gibco), 2 mmol/L L-glutamine (Gibco) and non-essential amino acids (Gibco). The cells were maintained at 37 ℃ in a humidified incubator containing 5 % CO_2. Insect cell Sf-9 was maintained in TNM-FH medium (Sigma) supplemented with 10 mL/L FBS for baculovirus generation, amplification and titration according to standard protocols[17]. The viruses were not concentrated by ultracentrifugation. The titer of Bac-GDH was 8.3×10^8

plaque forming units per ml.

Baculovirus transduction

A simple process eliminating the need for virus ultracentrifugation[18] was adopted for transduction. Briefly, BHK cells cultured in 6-well plates (5×10^5 cells per well) were washed with PBS, and then transduced simply by adding 100 μL unconcentrated virus solution and 400 μL PBS. The plates (or dishes) were shaken on a rocking platform for 6 h at 25 ℃. After the virus incubation, the virus solution was aspirated. The wells were replenished with 2 mL complete medium with or without sodium butyrate and incubated at 37 ℃. The cells grown on 10-cm dishes were transduced similarly, except that larger volumes of virus (800 μL) and PBS (3.2 mL) were used.

Western blot analysis

BHK cells were singly transduced by Bac-GDH (or Bac-GD), or co-transduced by Bac-GDH and Bac-GB. To detect intracellular L-HDAgH (or L-HDAg), the cells were harvested at 2 dpt (days post-transduction) and resuspended in TE buffer containing phenylmethylsulfony lfluoride (PMSF). The proteins were released by sonication on ice and separated from debris by centrifugation. To detect secreted HBsAg and L-HDAgH, virus-like particles released into conditioned medium from co-transduced cells were concentrated by 200 g/L sucrose-cushioned ultracentrifugation as described previously[13]. The pellet was resuspended in TE buffer for Western blot. The intracellular or extracellular samples were resolved on 120 g/L gels and transferred onto nitrocellulose membranes. The L-HDAgH (or L-HDAg) was probed by human anti-HDV sera[19] or mouse anti-His_6 mAb (Amersham Biosciences). The HBsAg was probed by anti-HBsAg mAb (A10F1, a kind gift from Prof. S.C. Lee, National Taiwan University). The secondary antibodies were AP-conjugated goat anti-human or anti-mouse IgG (Kirkegaard and Perry Laboratories). The bands were developed using BCIP/NBT reagent (Sigma). The densitometry was performed by scanning the bands on the membranes and analyzing by Scion Image Shareware (Scion Corporation). For estimation of the product yield, serially diluted purified protein was used as the standard and analyzed by scanning densitometry.

Flow cytometry

The transduced cells were harvested by trypsin/EDTA, washed and resuspended in PBS for flow cytometry (FACSCalibur, Becton Dickinson) analysis according to the manufacturer's instruction. The percentage of cells emitting fluorescence (% GFP + cells) and mean fluorescence intensity (MFI) of each sample were measured 3 times by counting 10 000 cells in each measurement. The mean± standard deviation (SD) of 3 independent experiments are presented as arbitrary units (au).

Immunofluorescence labeling

BHK cells were seeded onto 22 mm×22 mm glass slides and transduced in duplicate by Bac-GDH at a multiplicity of infection of 280. The cells were fixed at 24 h post-transduction (hpt) with methanol/acetone (1:1) for 5 min at -20 ℃, rinsed with PBS and then blocked with PBS containing 20 g/L bovine serum albumin (BSA) for 30 min at 37 ℃. For the detection of subcellular localization, L-HDAgH was probed with 150 μL anti-HDAg mAb (HP6A1, 1:300 dilution)[16] for 1 h at 37 ℃ and washed with PBS 3×5 min. L-HDAgH was subsequently labeled using Cy3-conjugated goat anti-mouse IgG (1:25 dilution, Sigma). For simultaneous nuclear staining, SYBR green I (1: 40 000 dilution, BioWhittaker) was added together with the secondary antibody. For isoprenylation analysis, L-HDAgH was first probed with primary antibody solution containing HP6A1 and anti-farnesyl mAb (Sigma) for 1 h at 37 ℃, followed by the labeling with secondary antibody solution containing FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit mAb (Jackson ImmunoResearch) for 1 h at 37 ℃. The slides were washed with PBS 3×5 min, mounted using glycerol/PBS (3:1) mounting solution and observed by a confocal microscope (TCS SP2, Leica, Germany).

Protein purification

The His_6-tagged L-HDAg was purified by immobilized metal affinity chromatography (IMAC) under denaturing conditions. The cells were resuspended in 10 ml binding buffer A (20 mmol/L Tris-HCl, 500 mmol/L NaCl, 8 mol/L urea and 10 mL/L PMSF, pH 7.8), sonicated for 5 min and centrifuged (12000 r/min for 30 min). After centrifugation, the supernatant was loaded cyclically for 1 h at room temperature to the Fast-flow Protein Liquid Chromatography (ÄKTA FPLC, Amersham Biosciences) system in which the column contained 5 mL Ni^{2+}-coupled Chelating Sepharose Fast Flow resin. Bound proteins were eluted by a mixture of elution buffers A and B (20 mmol/L Tris-HCl, 500 mmol/L NaCl, 500 mmol/L imidazole, pH 7.8) at 1 mL/min at 4 ℃. The FPLC system generated step gradients by varying the volume ratios of buffers A and B so that the urea concentration decreased while the imidazole concentration increased stepwise. The eluate was collected in 1.5 mL fractions and then analyzed by Western blots and SDS-PAGE.

RESULTS

Confirmation of protein expression

The recombinant baculoviruses designed to express L-HDAg with or without His_6 tags were constructed and designated Bac-GDH and Bac-GD (Figure 1). To confirm the correct expression of L-HDAgH in BHK cells, the cells grown on 10-cm dishes were transduced by Bac-GD or Bac-GDH, harvested at 2 dpt and the cell lysates were analyzed by Western blot. Figure 2A depicts that the mock-transduced cells expressed no protein recognized by the anti-HDV serum, while the Bac-GD-transduced cells expressed a protein whose molecular mass (27 kd) corresponded to that of authentic L-HDAg, indicating the specificity of the serum. Similarly, the Bac-GDH-transduced cells expressed a protein recognized by the antibody, but with a slightly higher molecular mass (about 31 kd), thus suggesting the fusion of the His_6 tag to L-HDAg. The identities of the proteins were further

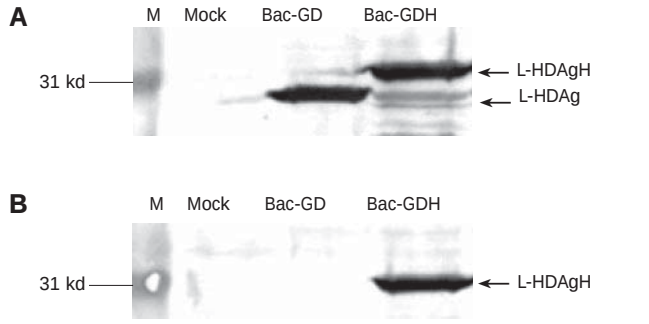
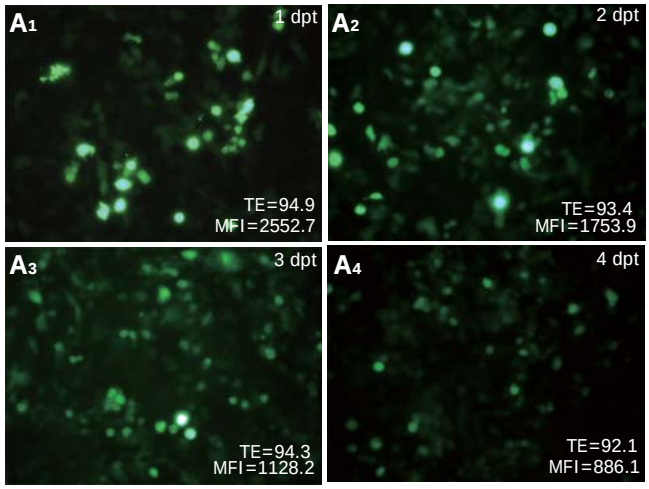


Figure 2 Western blot analysis of proteins expressed by mock-, Bac-GD- or Bac-GDH-transduced BHK cells. **A**: Anti-HDV sera; **B**: Anti-His$_6$ mAb.



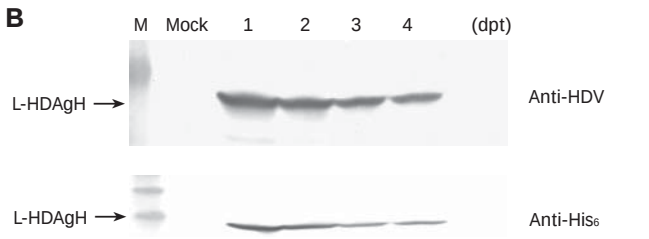


Figure 3 A: Variation of the transduction efficiency (TE) over time post-transduction. **B**: Time-course profile of L-HDAgH expression. Western blot using anti-HDV sera and anti-His6 mAb.

confirmed by analysis of the lysates using anti-His$_6$ mAb (Figure 2B). As expected, no His$_6$ tagged proteins were found in the mock- or Bac-GD-transduced cell lysates. In contrast, the 31 kd protein expressed by Bac-GDH was clearly visible. Figures 2A, B collectively confirme the expression of His$_6$ tagged L-HDAg in the BHK cells.

Transduction efficiency and time-course profile

To determine how efficiently the virus could transduce BHK cells and how the protein accumulation varied over time post-transduction, BHK cells were transduced and photographed using the fluorescence microscope. The percentages of GFP+ cells and the mean fluorescence intensities (MFI) were monitored over time by flow cytometry. As shown in Figure 3A, the percentage of GFP+ cells was up to 95 % at 1 dpt and remained higher than 90 % for 4 d, but the mean fluorescence intensities declined over time. Figure 3B shows the time-course profile of L-HDAgH expression and depicts that the expression reached the maximum rapidly at 1-2 dpt. The protein concentration declined slightly thereafter, but remained high at 4 dpt.

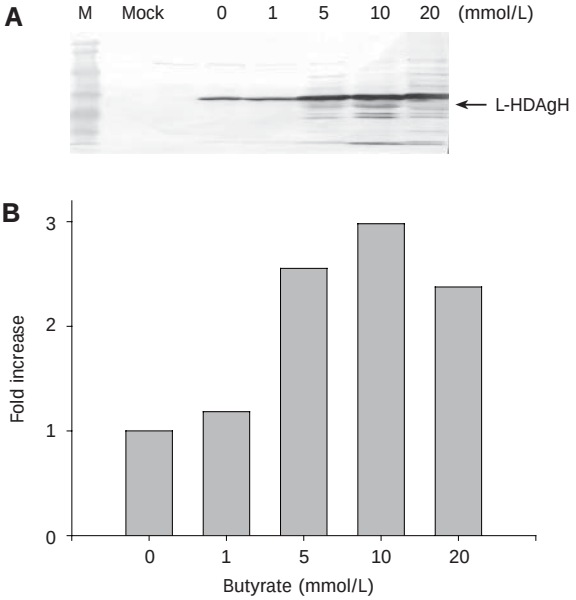


Figure 4 Effects of sodium butyrate on L-HDAgH expression. **A**. Western blot; **B**. The expression levels.

Enhancement of protein expression

To enhance the L-HDAgH expression, the cells were transduced on 10-cm dishes as described above, but varying concentrations of sodium butyrate, a histone deacetylase inhibitor shown to loosen the condensed chromosome and up-regulate the protein expression in mammalian cell system[20, 21], were supplemented to the medium after transduction. The cells were harvested at 2 dpt and the lysates were analyzed by Western blot. Figure 4A depicts a clear dependence of the L-HDAgH expression on the sodium butyrate concentrations. The scanning densitometry quantitatively revealed an about 3-fold enhancement in L-HDAgH expression by 10 mmol/L sodium butyrate, compared to the control without sodium butyrate (Figure 4B). The highest protein yield was roughly estimated to be about 19 pg/cell by scanning densitometry using serially diluted purified protein as the standard. At a higher concentration (i.e. 20 mmoL/L), the overall L-HDAgH expression dropped slightly probably due to high cytotoxicity associated with sodium butyrate[22].

Characterization of L-HDAgH

To determine whether baculovirus transduction and protein overexpression affected the cell physiology and deterred the correct protein localization, BHK cells were transduced by Bac-GDH, labeled by SYBR Green I and anti-HDAg mAb and examined by confocal microscopy. As shown, the nuclei were clearly marked by SYBR Green I (Figure 5A) while L-HDAgH was marked by clusters of

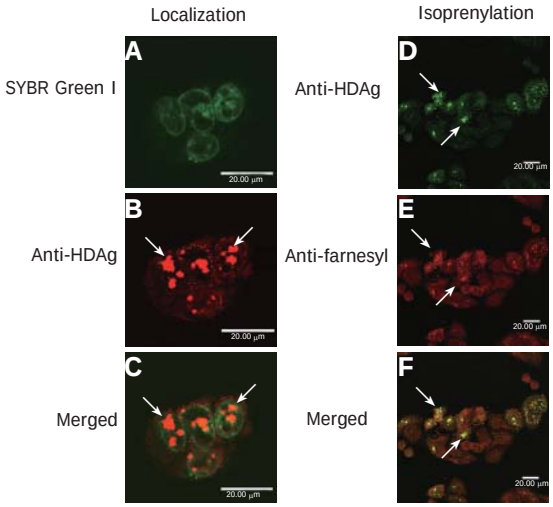


Figure 5 Subcellular localization and isoprenylation of L-HDAgH by immunofluorescence labeling. **A**: the presence of nuclei labeled by SYBR green I; **B**: L-HDAg detected using Cy3-conjugated goat anti-mouse IgG; **C**: merged photograph of A and B; **D**: the presence of L-HDAgH labeled by FITC-conjugated mAb; **E**: The presence of isoprenylation marked by TRITC-conjugated mAb; **F**: merged photograph of D and E. Bars, 20 μm.

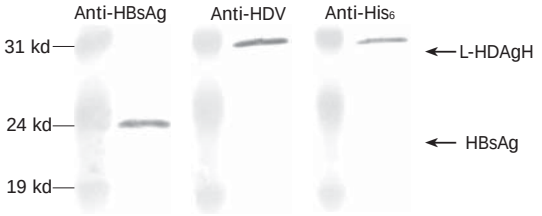


Figure 6 Western blot using anti-HBsAg, anti-HDAg and anti-His$_6$ antibodies.

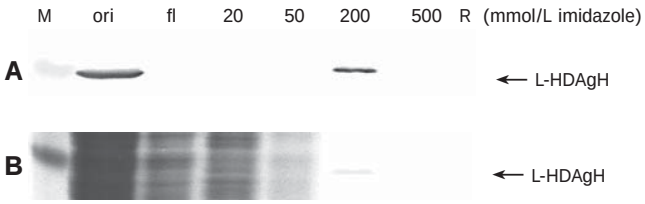


Figure 7 Western blot (**A**) and SDS-PAGE(**B**). M, marker; ori, original sample; fl, flow through; R, samples stuck in the resin. The numbers indicate the imidazole concentration at which the sample was collected.

red dots (Figure 5B). The merged photograph (Figure 5C) clearly illustrates the co-localization of L-HDAgH with the nuclei, confirming that the expressed L-HDAgH was localized within the nuclei.

To evaluate whether L-HDAgH was properly isoprenylated, Bac-GDH-transduced BHK cells were subject to immunofluorescence double labeling using anti-HDAg and anti-farnesyl mAb. Figure 5D shows the green dots marking the presence of L-HDAgH using anti-HDAg mAb, while Figure 5E illustrates the detection of isoprenylation using anti-farnesyl mAb as evidenced by the red dots. The merged photograph (Figure 5F) clearly illustrates the co-localization of both red and green dots, indicating that the baculovirus-expressed L-HDAgH was isoprenylated.

Since the assembly of HDV virions or virus-like particles (VLP) is an important function of L-HDAg, whether L-HDAgH remained capable of assembling into VLP was investigated. It has been shown that L-HDAg along with HBsAg was necessary and sufficient for the assembly of HDV VLP[13], thus BHK cells were co-transduced by Bac-GB and Bac-GDH. The conditioned medium was collected, concentrated and analyzed by Western blot. Figure 6 shows that the pellet contained proteins recognized by anti-HBsAg, anti-HDAg and anti-His$_6$ antibodies, confirming the simultaneous secretion of both HBsAg and L-HDAgH into the medium. Because L-HDAgH alone was localized within the nucleus, the co-secretion proved the assembly and secretion of HDV VLP[23-25].

Purification of L-HDAgH

Thanks to the fused His$_6$ tag, L-HDAgH was purified by IMAC. Under the native conditions, however, the protein binding to the resin was very weak (not shown). Therefore, the protein was denatured by 8 mol/L urea and purified under denaturing conditions. The Western blot (upper panel, Figure 7) shows that the flow through contained no L-HDAgH, indicating significantly improved binding. The proteins were then eluted with a mixture of buffers A and B with decreasing urea concentration and increasing imidazole concentration. The L-HDAgH was eluted by 200 mmol/L imidazole buffer (4.8 mmol/L urea). The purity was 7 % as revealed by SDS-PAGE (lower panel, Figure 7).

DISCUSSION

As mentioned above, L-HDAg may represent an excellent vaccine candidate, but characterization and evaluation of the immunological properties of L-HDAg have been impeded due to the lack of a proper method for efficient expression and purification. Although attempts to express L-HDAg using *E coli* or baculovirus/insect cell systems have been made, both systems are not suited for L-HDAg expression because the former lacks post-translational modification while the latter results in rapid degradation of L-HDAg after 2 d post-infection[26]. Besides, L-HDAg expressed in insect cells induces cell cycle arrest[27]. The integration of L-HDAg gene into the chromosome of mammalian cells for stable expression may offer another option, but has not been reported probably because L-HDAg is a nuclear protein, the accumulation of which can result in significant cytotoxicity. Transient expression in mammalian cells mediated by other virus systems (e.g. vaccinia virus) may be possible as well, but these viruses lead to cell death and lysis, thus potentially undermining the post-translational modification at later stage. Owing to these restrictions, L-HDAg expression is mainly achieved by transfecting plasmids expressing L-HDAg into hepatoma cells (e.g. Huh-7 and HepG2). The plasmid transfection into hepatocytes, however, is notorious for

its low efficiency and low expression level, consequently, highly sensitive detection methods such as isotopic labeling and chemiluminescence enhancement are required to detect the expression.

To express functional L-HDAg efficiently and rapidly for research purposes, the baculovirus/mammalian cell system was employed. Using PBS as the surrounding solution, the efficiency of baculovirus-mediated gene delivery into BHK cells was up to 94 % (Figure 3A), which far exceeded 20 %-30 % normally achieved by plasmid transfection. The detection of L-HDAgH using anti-HDV sera and anti-His_6 mAb (Figure 2) confirmed that the expressed L-HDAgH possessed the fused His_6 tag and was similar in size and antigenic properties to those of authentic proteins. The expression level was apparently higher than that can be obtained using plasmid transfection, as evidenced by the easy detection using BCIP/NBT as the color developing reagent, which was significantly less sensitive than chemiluminescence enhancement. Under the transcriptional control of CMV-IE promoter, the L-HDAgH expression peaked at day 1-2 and gradually declined thereafter, which probably stemmed from cell proliferation and attenuation of promoter activity. Albeit this, the expression level could be up-regulated by sodium butyrate and reached the maximum (at about 19 pg/cell) at 10 mmol/L sodium butyrate (Figure 4A).

Besides the high transduction efficiency and high expression level, Figures 5A-C illustrate that L-HDAgH formed speckles localized within the nuclei of BHK cells, which is the subcellular location of authentic L-HDAg[28]. The formation of nuclear speckles is a sign of isoprenylation[29] and could have stemmed from 1) the formation of dimmers or multimers of L-HDAg between the coiled-coiled domains or 2) the interaction of L-HDAg with host proteins such as nucleolin and delta-interacting protein A[30]. The formation of aggregates has been previously observed for L-HDAg expressed in hepatoma cell lines via plasmid transfection[28], and explains the low binding of L-HDAgH to Ni^{2+}-coupled resin under native conditions and the low purity in subsequent purification steps. Further improvement in the purification process is required and is currently underway. Furthermore, Figures 5D-5F revealed that L-HDAgH underwent proper isoprenylation, which is a prerequisite for the interaction of L-HDAg with HBsAg (and hence the virion formation). The Western blot analysis of concentrated pellets (Figure 6) further confirmed the assembly and secretion of HDV VLP. The retention of isoprenylation as well as formation of VLP implied that the biological function of L-HDAgH was not impaired by the His_6 tag[31, 32].

In summary, our data solidly confirm that the overexpression of His_6 tagged L-HDAgH in baculovirus-transduced mammalian cells does not hamper the proper localization and processing. The data also suggest the maintenance of accurate structure and appropriate biological functions and hence validate the use of baculovirus/mammalian cell system for the production of eukaryotic proteins requiring extensive post-translational modifications. This is partly attributed to the fact that baculovirus does not cause cell lysis or induce cytotoxicity. Additionally, the baculovirus system enables protein production in BHK cells which is one of the few cell lines approved for biopharmaceutical production. Moreover, the transduction protocol eliminates the need for virus concentration by ultracentrifugation so that transduction can be carried out in the bioreactor simply by incubating the virus solution/PBS with the cells, which otherwise is difficult for plasmid transfection. Since process scale-up of BHK culture has been well established, large scale production of L-HDAg can be made possible simply by transferring this process to a bioreactor (e.g. a packed bed bioreactor).

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BASIC RESEARCH

Relaxin prevents the development of severe acute pancreatitis

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Abstract

AIM: To investigate the severity of acute pancreatitis (AP) is associated to the intensity of leukocyte activation, inflammatory up-regulation and microcirculatory disruption associated to ischemia-reperfusion injury. Microvascular integrity and inhibition of pro-inflammatory mediators are key-factors in the evolution of AP. Relaxin is an insulin-like hormone that has been attributed vasorelaxant properties via the nitric oxide pathway while behaving as a glucocorticoid receptor agonist.

METHODS: AP was induced by the bilio-pancreatic duct-outlet-exclusion closed-duodenal-loops model. Treatment with relaxin was done at different time-points. Nitric oxide synthase inhibition by L-NAME and glucocorticoid receptor (GR) blockage by mifepristone was considered. AP severity was assessed by biochemical and histopathological analyses.

RESULTS: Treatment with relaxin reduced serum amylase, lipase, C-reactive protein, IL-6, IL-10, hsp72, LDH and 8-isoprostane as well as pancreatic and lung myeloperoxidase. Acinar and fat necrosis, hemorrhage and neutrophil infiltrate were also decreased. ATP depletion and ADP/ATP ratio were reduced while caspases 2-3-8 and 9 activities were increased. L-NAME and mifepristone decreased the efficiency of relaxin.

CONCLUSION: Relaxin resulted beneficial in the treatment of AP combining the properties of a GR agonist while preserving the microcirculation and favoring apoptosis over necrosis.

Key words: Acute pancreatitis; Relaxin; Nitric oxide; Glucocorticoid receptor; Necrosis; Apoptosis

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INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory disorder associated with premature activation and increase in pancreatic enzymes in blood and urine that may progress to implicate other organs such as kidney, lungs and liver[1]. Inflammatory mediators induce complications that can range from local to systemic evolving to a severe form with high morbi-mortality[2].

The peri-vaterian duodenum (PV-D) has an extremely rich autonomic duodenum-pancreatic innervation therefore endoscopic maneuvers, gallstones, biliary sludge and/or hemobilia migrating and impacting on the peri-vaterian duodenum stimulate primary sensory neurons to unchain autonomous-arc-reflexes (AARs) responsible for the initial acute pancreatic neurogenic inflammation that may range from local and edematous to regional and necrotizing pancreatitis[3-6]. These autonomic nerve fibers are the starting point of autonomic-arc-reflexes which may integrate in the celiac ganglia complex or in the bulbar-hypothalamic autonomous nucleus to end in the intrapancreatic ganglion[6]. Therefore irritative stimuli induce impulses that travel through thin, unmyelinated, capsaicin-sensitive (type C) nerve fibers to generate in the somata of the afferent neurons vasoactive peptides such as substance P, neurokinin-A, VIP, SOM and DYN[7]. These sensory peptides synthesized in the somata are then transported to the nerve peripheral endings where they are released. Substance P and other sensory peptides can activate mast-cells that will liberate histamine and pro-inflammatory factors thus recruiting leukocytes that will exacerbate the inflammatory response associated to vasodialtation, edema, increased vascular permeability and neutrophil infiltration associated to this neuroimmune-

inflammatory interaction observed in the neurogenic inflammation[7].

Over-stimulation of receptors in the peri-vaterian duodenum trigger autonomic-arc-reflexes that induce a sympathetic hypertone followed by cathecolaminic discharges from nerve endings in the pancreas that affect capillaries and blood vessels leading to microcirculatory disturbances responsible for ischemia/ reperfusion injury associated to synthesis and release of pro-inflammatory factors together with leukocyte recruitment and activation that correlate with intracellular enzyme activation[7]. Under these circumstances the systemic inflammatory response syndrome (SIRS) may end in a multiorgan dysfunction syndrome (MODS) characterized by metabolic and homodynamic alterations, multifocal organic dysfunction and pluriparenchymatose failure[8]. The bilio-pancreatic-duct outlet exclusion closed duodenal loops (BPDOE-CDLs) model, that we have previously described and validated, mimics these circumstances[6].

Relaxin is a peptide hormone of ovarian origin that belongs to the insulin superfamily[9]. It has been reported that relaxin offers protection for reperfusion-induced cardiac injury increasing coronary blood flow favoring blood supply to the ischemic myocardium while it exerts a relaxant action on smooth muscle cells[10]. It has been shown that relaxin up regulates nitric oxide synthase (NOS) II expression thus inducing nitric oxide mediated vasodilatation by a controlled activation of endogenous nitric oxide biosynthesis[11]. By regulating nitric oxide relaxin inhibits neutrophil extravasation, adhesion, recruitment and activation[12]; decreases the expression of chemokines and adhesion molecules[13]; inhibits histamine release by mast cells[14]; depresses platelet aggregation[15]; alters Ik-B phosphorylation and degradation diminishing NF-kB activation and binding to DNA and enhances the activation of the glucocorticoid receptor (GR)[16]. Therefore, via the nitric oxide pathway relaxin exerts vaso-relaxant, anti-thrombotic and anti-inflammatory properties.

Relaxin has also been shown to bind and stimulate the glucocorticoid receptor that when activated will translocate to the nucleus where it inhibits NF-kB and AP-1 by transrepression and stimulates transcription of glucocorticoid response elements (GREs) of DNA inducing transcription of responsive genes by transcativation[16] . As an agonist of the glucocorticoid receptor relaxin will then possess anti-inflammatory properties inhibiting pro-inflammatory molecules.

Our aim was to investigate the influence of relaxin on the evolution of acute pancreatitis considering the relevance of the nitric oxide pathway and glucocorticoid receptor stimulation. Pancreatic, metabolic, kidney, liver and severity indicators were assessed.

MATERIALS AND METHODS

General concepts

The Bilio-pancreatic-duct-outlet-exclusion closed-duodenal-loops model (BPDOE-CDL) model was carried out in male inbred adult Wistar rats, weighing 250 - 350 g, free of pathogens, under controlled environmental conditions (room-temperature, 20-25 ℃; 12:12 light-dark cycle), with free access to standard rodent chow and water. The study was performed in accordance with national guidelines for the use and care of laboratory animals and was approved by the local animal care and use committee. Rats were randomly assigned to one of the three series: basal, control or experimental. Each of these series was divided into groups of 16 rats.

The animal series comprised eleven groups. *Group 1* (reference baseline values), consisted of un-fasted, healthy animals that did not undergo any surgery or treatment. *Group 2* (surgery control), only underwent the surgical maneuvers involved in triggering AP. *Group 3* (AP, BPDOE-CDLs model) acute pancreatitis was triggered by filling the closed-duodenal-loops at constant pressure with 8% sodium taurocholate [SIGMA, USA] and 200 uL of methylene blue to corroborate duodenal-reflux absence[6]. *Group 4* (PBS vehicle control and BPDOE-CDLs) acute pancreatitis was induced as for Group 3 and 100 uL PBS were injected sc 1h before and 4 h after the surgery. *Group 5* (LN+RLX, non-selective NOS inhibitor and relaxin) L-NAME (N^G-nitro-L-arginine methyl ester [SIGMA, USA]) at 10 mg/kg and 5 mg/kg and relaxin [Phoenix Pharmaceuticals Inc, USA] at 2.5ug/kg were administered sc 1h before and 4h after triggering acute pancreatitis. *Group 6* (MIF+RLX, GC receptor antagonist and relaxin) received mifepristone [SIGMA, USA] at 3 mg/kg and relaxin at 2.5 ug/kg sc 1h before and 4h after unchaining acute pancreatitis. *Group 7* (LN+MIF+RLX, non-selective NOS inhibitor associated to GC receptor antagonist and relaxin) L-NAME at 10 mg/kg and 5 mg/kg, mifepristone at 3 mg/kg and relaxin at 2.5ug/kg were administered sc 1h before and 4h after triggering acute pancreatitis. *Group 8* (RLX 1h pre BPDOE-CDLs) received 5ug/kg sc of relaxin 1h before the surgical maneuvers. *Group 9* (RLX 1h post BPDOE-CDLs) was injected with 5 ug/kg sc of relaxin 1h after the procedure. *Group 10* (RLX 1h pre and 4h post BPDOE-CDLs) was administered Relaxin at 2.5 ug/kg sc 1h before and 4h after unchaining acute pancreatitis. *Group 11* (RLX 4h post BPDOE-CDLs) was treated with 5 ug/kg sc of relaxin 4h after triggering acute pancreatitis. L-NAME and mifepristone were dissolved in saline solution, relaxin was dissolved in PBS. *Group 12* (RLX 12h &1h pre and 4h post BPDOE-CDLs) received relaxin at 5 ug/kg sc 12 h before the surgery and 2.5 ug/kg sc 1h prior to the procedure and a second administration 4h after it.

Experimental procedures

Bilio-pancreatic-duct-outlet-exclusion closed-duodenal-loops model (BPDOE-CDLs) We described the BPDOE-CDLs model in detail Figure 1, (reproduced with permission granted by the Journal of Pancreatology and S. Karger AG, Basel)[6]. The normally fed animals were anesthetized with sodium pentothal (5 mg/100 g) (Thiopental, Abbott, USA). A median laparotomy was done to exteriorize the antrum-duodenum-pancreas, and a double-tube double-hole intra-duodenal catheter was inserted, through which saline solution (3 mL) was instilled. Two closed-duodenal-loops were prepared: first two proximal pre-pyloric ligatures and one post-pyloric just above the common-bilio-pancreatic duct outlet

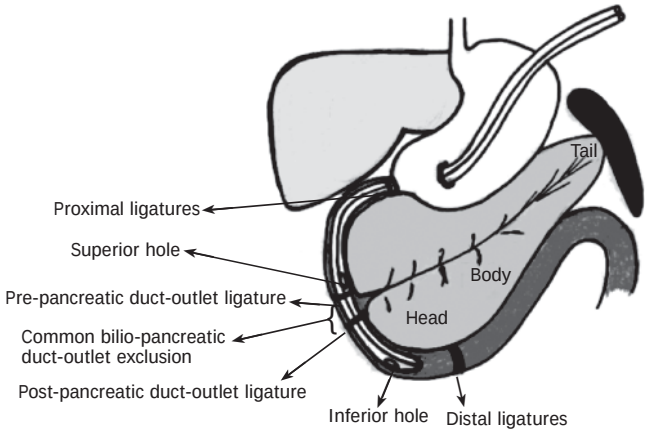

Figure 1 BPDOE-CDL model to trigger acute pancreatitis. Two closed-duodenal-loops were created in such a way that the entrance of the common bilio-pancreatic duct-outlet was absolutely excluded. To double check the absence of duodenal-reflux content or duodenal leakage, methylene blue was added to the sodium tauracholate which was instilled into the peri-vaterian duodenum through the superior and inferior holes (reproduced with permission granted by the Journal of Pancreatology and S. Karger AG, Basel)[6].

were placed. For the second loop, a ligature was placed just below the common-bilio-pancreatic-duct and two more ligatures were placed at the distal end of the peri-vaterian duodenum, thus excluding the common-bilio-pancreatic-duct outlet. Each closed-duodenal-loop was filled with 8% sodium taurocholate (1 mL) and 200 uL of methylene blue at constant pressure, following a 45-minute observation period. The closed-duodenal-loop content was aspirated, the ligatures removed and the laparotomy closed. The animal was then placed in an individual cage with microenvironmental temperature control and allowed to recover. After 12 h, the rat was exsanguinated under anesthesia by aortic puncture and a blood sample was obtained for biochemical tests. All procedures were done with aseptic techniques. If, despite exclusion of the common-bilio-pancreatic-duct, duodenal-content filtered through the ligatures and refluxed towards the bilio-pancreatic ductal tree, as evidenced from blue coloring of the pancreas, the rat was excluded from the study.

Macroscopic examination, dissection and weighing with a precision scale (Microgram-Series BH 300) of the pancreas were performed. The gland was subsequently divided into cephalic and splenic segments[6,17,18].

The liver, duodenum, kidneys, suprarenal glands, spleen and lungs were examined and dissected. Each organ was divided into smaller portions, one was fixed in formol-buffer and stained with hematoxylin-eosin, the others were wrapped individually in aluminum foil and preserved at -70 ℃. The cephalic and splenic segments of the pancreas and the lungs were studied in more detail.

Amylase and lipase activity measurement and protein quantification in pancreatic tissue Assays were done on each segment; the tissue was homogenized on ice in 2 mL of extraction buffer (20 mmol/L Tris-HCL pH 7.6-[Sigma, USA], 0.3 mol/L NaCl-[Merck, Germany], 0.5% Triton X-100-[Sigma, USA]; distilled water). Proteins were quantified in the homogenate according to the method described by Lowry[19]. The homogenate was centrifuged at 10000 r/min for 15 min at 4 ℃. The supernatant was assayed for amylase (kinetic method at 405 nm with defined substrate at 25 ℃-[Wiener, Arg.]) and lipase (turbidimetric method UV color at 25 ℃-[Randox, UK]).

Myeloperoxidase (MPO) activity in pancreas and lung tissue MPO, an enzyme found in neutrophils that represents the 5% of its dry weight, catalyzes the oxidation of electron donors (eg.halides) by hydrogen peroxide[20]. MPO was measured in both pancreatic segments and in lung tissue as a biochemical indicator of neutrophil infiltrate. To determine MPO activity the tissue was homogenized on ice with 1 mL hexadecyltrim ethylammonium bromide (HTAB) (0.5 % HTAB in 50 mmol/L phosphate buffer-pH 6.0). The homogenate was sonicated during 10 s at 200 W, and then centrifuged at 40000 r/min for 15 min at 4 ℃. MPO was assayed on the supernatant: 0.1 mL of the sample was combined with 2.9 mL of 5 mmol/L phosphate buffer, (pH 6.0, 0.167 mg/mL o-dianisidine hydrochloride and 0.0005% hydrogen peroxide). The change in absorbance at 460 nm was measured with a Clinicon 4010 spectrophotometer. One unit of MPO (UMPO) activity was defined as the amount that could degrade 1 μmol of peroxide per minute at 25 [21].

Caspase 2-3-8 and 9 activities in pancreas, liver and lung tissue Caspases 2-3-8 and 9 are early indicators of apoptosis in mammalian cells[22]. The CasPASE™ apoptosis activity assay kit [Genotech, MO-USA] is based on detecting the chromogenic and fluorogenic resulting molecule 7-amino-4-trifluromethyl cumarin (AFC) cleaved from the synthetic substrate, Z-VAD-fluoromethyl ketone (FMK) irreversibly and selectively inhibits the reaction. The excitation and emission wavelengths of AFC in a fluorometer are 360 nm and 510 nm respectively. Frozen tissues were homogenized at 4 ℃ in chilled lysis buffer and the lysates were then centrifuged at 15000 g for 30 min at 4 ℃, caspase activity was assessed on the supernatants. To calculate specific enzyme activity equal aliquots of supernatant were tested in presence of the Z-VAD-FMK inhibitor. Results were expressed as the specific cleavage of AFC per mg of protein per minute considering the nonspecific product formation coming from the substrate plus the inhibitor. All reagents were supplied by the kit and the assays were performed according to the manufacturer's instructions.

ATP and ADP/ATP ratio measurements in pancreas, liver and lung tissue ATP depletion allows to detect apoptosis, and when it is associated with the percentage of ATP loss and changes in the ADP/ATP ratio apoptosis can then be conveniently differentiated from necrosis[23]. Light formed from ATP and luciferin in the presence of luciferase can be measured at 560 nm. The tissue was homogenized in TCA (trichloroacetic acid) 6% for 1 min and centrifuged at 6000 g for 5 min at 4 ℃. The TCA in the supernatant was neutralized and diluted to a final concentration of 0.1% with Tris-Acetate pH 7.75 buffer. ATP concentration expressed as nmol/L per mg of protein and ADP/ATP concentration ratio were measured in the supernatant using the ENLITEN® ATP Assay System Bioluminescence Detection Kit [Promega, WI-USA] and ApoSENSOR™ ADP/ATP ratio assay kit (Alexis-Corp, Switzerland) respectively according to the manufacturer's instructions.

Extraction-purification-quantification of DNA The total mass of DNA in the pancreas, and lung tissue was used to standardize the units of enzymatic activity since it did not show significant quantitative variations in the BPDOE-CDL model.

The DNA was extracted and purified from tissue from both pancreatic segments and lung according to the manufacturer's instructions (Wizard Genomic- [Promega, USA]). Double stranded DNA concentration was measured at 260 nm.

Histologic evaluation Pancreatic tissue was graded on a scale of 0-4 each for: edema, hemorrhage, leukocyte infiltration, acinar necrosis and fat necrosis. Lung tissue was graded using a scale of 0-4 for leukocyte infiltration. Histologic changes were graded in a blinded manner by two independent observers counting frequency of foci per field seen at 40X (absent = 0, mild = 1, moderate = 2, severe = 3, overwhelming = 4).

Biochemical assays in blood samples *Loss of blood and hemoconcentration* were evaluated by the hematocrite. The *metabolic profile* consisted of glucose (enzymatic method in serum at 37 ℃-[Wiener, Arg.]), uric acid (enzymatic method in serum at 37 ℃-[Wiener, Arg]), proteins (enzymatic method in serum at 37 ℃-[Wiener, Arg.]) and calcium (direct colorimetric method in serum at 20 ℃-[Wiener, Arg.]). The *kidney profile* comprised urea (enzymatic method in serum at 37 ℃-[Wiener, Arg.]) and creatinine (end-point colorimetric method in serum at 37 ℃-[Wiener, Arg]). The *pancreatic profile* involved amylase (kinetic method at 405 nm with defined substrate in serum at 37 ℃-[Wiener, Arg.]) and lipase (UV turbidimetric method in serum at 37 ℃-[Randox, UK]). The *liver profile* consisted of AST and ALT (IFCC optimized UV method in serum at 37 ℃-[Wiener, Arg.]), bilirubin (colorimetric method to determine total and direct bilirubin in serum at 25 ℃, indirect bilirubin that results from the difference between them-[Wiener, Arg.]) and alkaline phosphatase (optimized DGKC-SSCC kinetic method at 405 nm in serum at 37 ℃-ALP 405-[Wiener, Arg.]) *Cellular injury* was assessed by LDH (DGKC optimized kinetic method in serum at 37 ℃-[Wiener, Arg.]). The *profile for cellular stress and inflammatory response associated to severity* involved leukocyte count, quantitative C reactive protein (immunoturbidimetric method in serum at 37 ℃ -PCR Latex Turbitest-[Wiener, Arg.]), heat shock protein (hsp) 72 (ELISA method for rat HSP 72 in plasma and serum-[StressGen Biotechnologies, USA)], IL-6 and IL-10 (ELISA technique in serum-[Assay Designs,USA]) and 8-Isoprostane (EIA method in plasma-[Cayman, USA]).

Statistical analysis

Biochemical assays in blood and organs The ANOVA test was used to establish differences between groups. The Dunnet test compared the difference between each group in relationship to group 1 (reference baseline level in control animals). The Student-Newman-Keuls test carried out after the ANOVA compared the groups between them and in relationship to group 1, evaluating the existence of homogeneous groups. Results are expressed as mean ± standard deviation (SD). Differences were considered statistically significant with $P < 0.05$.

Histopathological score The ANOVA test with two fixed factors (group and place: cephalic and splenic) was followed by the Dunnet test and Student-Newman-Keuls test. Results are expressed as mean ± SD. Differences were considered statistically significant with $P < 0.05$.

Correlation between MPO and leukocyte infiltrate In both pancreatic segments and in lung tissue, the Spearman correlation coefficient Rho was calculated to examine the correlation between MPO and leukocyte infiltrate[24].

RESULTS

Biochemical assays in blood and other tissues together with histopathological studies evaluated the influence of relaxin on the evolution of acute pancreatitis. Association with L-NAME and/or mifepristone pondered on the weight of the nitric oxide pathway and glucocorticoid receptor stimulation involvement in acute pancreatitis modulation by relaxin. These results are described in Tables 1-4.

Pancreatic and general biochemical assays in blood

To evaluate if relaxin helped to maintain pancreatic, metabolic, kidney and liver function after the initiation of acute pancreatitis, pancreatic and general biochemical assays in blood were performed (Table 1).

Cholestasis was absent in all groups. Uric acid, alkaline phosphatase, total - direct and indirect bilirubin showed no significant differences among the mean values for all groups, the rest of the assays did.

Amylase and lipase showed a significant increase for homogenous groups (3-4) that developed a severe acute pancreatitis, group 7 also exhibited a marked raise although not to the same extent and the values of the enzymes for homogenous groups (5-6) were slightly lower than for group 7. Relaxin treatment 12& 1h before and 4h after BPDOE-CDLs (group 12) was similar to the one corresponding to 1h pre and 4h post pancreatic maneuvers (group 10). These treatments resulted in the least increase in pancreatic enzymes. These same trend was observed for glucose, urea, creatinine, AST and ALT. The abnormal reduction profile in calcium and serum proteins presented the same course as the pathological augmentation observed for pancreatic enzymes. The hematocrite value, considering group 2 as the reference group due to blood loss during surgery, indicated that homogenous groups (10-12), developing mild acute pancreatitis did not manifest the hemoconcentration observed for homogenous groups (3-4).

2- Severity indicators in blood

We next wanted to assess if the severity of acute pancreatitis was ameliorated by relaxin treatment measuring blood parameters (Table 2 & Figure 2A). The raise in leukocyte count correlated ($R = 803$, $P < 0.05$) with the expression of inflammatory IL-6 and liver synthesized acute phase protein CRP. The significant increase observed for homogenous groups (3-4) was followed in order by that developed in group 7 and then by homogenous groups (5-6); these values were markedly diminished for homogenous groups (10-12). Inhibition of either the nitric oxide pathway or glucocorticoid receptor stimulation

Table 1 Pancreatic and general biochemical assays in blood (mean ± SD)

Groups	Amylase IU/L	Lipase IU/L	Glucose mg/dL	Urea mg/dL	Creatinine mg/dL	Calcium mg/dL	Proteins g/L	AST IU/L	ALT IU/L	Hto %
1 Basal	1103±108	85±18	92±13	29±3	0.35±0.04	13.48±0.39	85.5±1.2	177±25	88±19	39±5
2 Surgery control	1198±114	98±10	95±10	38±5	0.48±0.11	13.22±0.35	82.7±0.8	205±21	99±21	31±4
3 AP	7915±159	5619±194	338±24	219±19	1.82±0.21	8.92±0.16	61.1±1.3	589±26	295±28	49±9
4 AP+PBS	7921±137	5628±201	341±19	217±15	1.81±0.16	8.94±0.20	61.2±2.2	591±25	299±28	49±8
5 Rlx+LN	7154±165	4982±203	246±23	183±22	1.58±0.22	9.37±0.18	65.5±1.8	544±30	268±19	47±6
6 Rlx+MIF	7162±171	4997±207	249±27	185±24	1.60±0.25	9.35±0.14	65.1±1.9	552±28	270±22	47±8
7 Rlx+LN+MIF	7548±175	5327±214	262±33	194±21	1.69±0.22	9.11±0.21	62.4±1.4	563±34	281±27	48±5
8 Rlx 1h pre	6052±169	3618±155	190±16	125±11	1.13±0.14	11.19±0.22	74.5±2.3	405±24	196±23	40±4
9 Rlx 1h post	6427±185	3910±183	218±15	146±15	1.24±0.19	10.91±0.19	71.1±1.9	452±37	211±27	45±4
10 Rlx 1h pre+4h post	5589±129	3161±142	166±18	97±8	1.02±0.17	11.98±0.24	79.4±1.6	382±29	178±23	42±5
11 Rlx 4h post	6896±192	4533±178	235±29	165±19	1.39±0.20	10.21±0.19	69.9±2.1	497±33	235±25	46±5
12 Rlx 12h&1h pre+4h post	5535±144	3153±121	162±22	95±10	1.00±0.15	12.03±0.29	79.8±1.4	395±33	171±25	43±5

In relationship to Reference Basal-Group 1, BPDOE-CDLs AP-Group 3 presents a significant difference in all the assays evaluated $P<0.05$. The pancreatic and general biochemical profile significantly improved with relaxin treatment particularly for Groups 10 &12 that corresponded to administration times 12h and/or 1h pre and 4h post surgery $P<0.05$. Homogenous groups for amylase and lipase:1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. HTO: hematocrite, Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

Table 2 Severity indicators assays in blood (mean ± SD)

Groups	Leukocytes nr/mL	CRP mg/dL	HSP 72 pg/mL	IL-6 pg/mL	IL-10 pg/mL	8-isoprostane pg/mL
1 Basal	6100±224	0±0	739±32	below detection limit	below detection limit	0.11±0.005
2 Surgery control	9856±272	3.3±1.02	1221±21	207±26	382±59	0.13±0.006
3 AP	26162±258	30.9±1.15	3396±64	1962±27	2216±94	0.55±0.011
4 AP+PBS	26145±294	30.6±1.21	3378±56	1956±32	2201±85	0.56±0.010
5 Rlx+LN	21223±207	21.7±1.24	2727±48	1792±28	2063±90	0.49±0.010
6 Rlx+MIF	21207±236	21.4±1.31	2765±52	1784±35	2052±78	0.48±0.009
7 Rlx+LN+MIF	23239±273	27.3±1.12	3023±39	1849±29	2113±122	0.50±0.008
8 Rlx 1h pre	17438±266	13.9±1.22	1924±49	1473±33	1695±72	0.37±0.010
9 Rlx 1h post	18776±248	16.4±1.52	2258±42	1601±25	1809±87	0.42±0.009
10 Rlx 1h pre+4h post	14527±285	10.8±1.44	1711±39	1098±32	1451±68	0.31±0.010
11 Rlx 4h post	19349±225	19.5±1.46	2506±38	1689±23	1975±84	0.46±0.008
12 Rlx 12h&1h pre+4h post	14542±295	10.9±1.27	1721±43	1105±28	1460±76	0.32±0.007

Severe AP was developed in groups 3 & 4. This severity was ameliorated by treatment with relaxin. In relationship to reference basal-group 1, BPDOE-CDLs AP in group 3 presented a significant difference in all the assays evaluated while treated groups 10 & 12 were the ones to exhibit the best prognosis with a significant improvement for all markers $P<0.05$.Homogenous groups for severity indicators :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

reduced the efficiency of relaxin treatment; inhibiting simultaneously both of them although leading to a more severe manifestation of the disease still showed better prognosis than that observed for homogenous groups (3-4) suggesting that there may be another mechanism by which relaxin may be also acting. Treatment with a single dose of relaxin 1h before, 1h after or 4h after unchaining acute pancreatitis although still beneficial was not as effective. Pro-inflammatory IL-6 and anti-inflammatory IL-10 exhibited the same profile as a consequence of the induction of the inflammatory response and attempt to counter-act it. Lipid peroxidation leading to elevated 8-isoprostane and synthesis of hsp72 aiming to protect from ongoing cellular stress followed the same trend.

Assays in pancreatic and lung tissue

We then wanted to investigate the enzymatic and inflammatory pancreatic and lung response (Table 3).

There were no significant differences between the mean value of the groups for DNA of the lung and the same pancreatic segment, but the mean values for the cephalic and splenic segments for each group were significantly different (data shown previously[6]).

The decrease in pancreatic amylase and lipase was inverse to their increase in blood. Pancreatic proteins showed a similar expression profile to that of pancreatic enzymes, the elevation for group 2 may result from the synthesis of acute phase pancreatic proteins.

Pancreatic and lung MPO activity reflecting neutrophil

Table 3 Assays in pancreatic and lung tissue (mean ± SD)

	Panc. Amylase IU/mg DNA ds		Panc. Lipase IU/mg DNA ds		Panc. Proteins mg/mg DNA ds		Pancreatic MPO UMPO/mg DNA ds		Lung MPO UMPO/mg DNA ds	Panc. Wet Weight mg/100 g body wt
Groups	H	B&T	H	B&T	H	B&T	H	B&T		
1 Basal	995±24	603±27	98±10	75±11	185±17	103±12	0±0	0±0	0±0	1310±19
2 Surgery control	927±19	576±22	81±11	66±10	206±19	122±14	0±0	0±0	0±0	1385±24
3 AP	263±17	181±14	26±9	16±8	115±10	57±10	10.64±0.39	7.15±0.11	3.85±0.13	2335±38
4 AP+PBS	261±20	185±26	25±9	15±9	114±11	58±10	10.59±0.31	7.22±0.09	3.83±0.12	2321±43
5 Rlx+LN	392±18	216±21	34±5	24±6	129±14	64±8	8.12±0.32	5.21±0.12	2.93±0.17	2159±40
6 Rlx+MIF	389±19	219±23	35±7	23±5	130±12	64±13	8.09±0.36	5.27±0.15	2.96±0.15	2142±48
7 Rlx+LN+MIF	315±15	202±12	32±8	19±4	121±10	60±8	9.01±0.42	6.16±0.10	3.10±0.14	2198±39
8 Rlx 1h pre	590±29	342±16	50±12	37±7	154±18	81±16	5.94±0.22	3.68±0.08	2.21±0.08	1798±33
9 Rlx 1h post	505±33	307±35	45±10	32±4	147±20	70±10	6.23±0.25	3.95±0.11	2.43±0.09	1967±39
10 Rlx 1h pre+4h post	679±31	387±18	60±9	43±8	165±23	90±11	5.79±0.19	3.59±0.09	2.06±0.10	1688±29
11 Rlx 4h post	439±22	273±24	39±9	27±9	135±15	66±7	7.05±0.28	4.45±0.09	2.64±0.14	2087±42
12 Rlx 12h&1h pre+4h post	683±26	392±17	59±8	44±9	167±21	89±10	5.78±0.21	3.54±0.03	2.07±0.09	1693±25

Pancreatic amylase and lipase decreased significantly in severe AP group 3, $P<0.05$. Treatment with relaxin induced a significant improvement with an increase in pancreatic enzymes and proteins $P<0.05$, while there was a decrease in UMPO. This amelioration of the disease was even more remarkable for groups 10 & 12 indicating that treatment 12 and/or 1h pre and 4h post BPDOE-CDLs procedure had a protective role. The increase in pancreatic wet weight in groups with severe AP as a result of edema was significant $P<0.01$. Homogenous groups for pancreatic amylase-lipase and MPO: :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$; lung MPO: :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

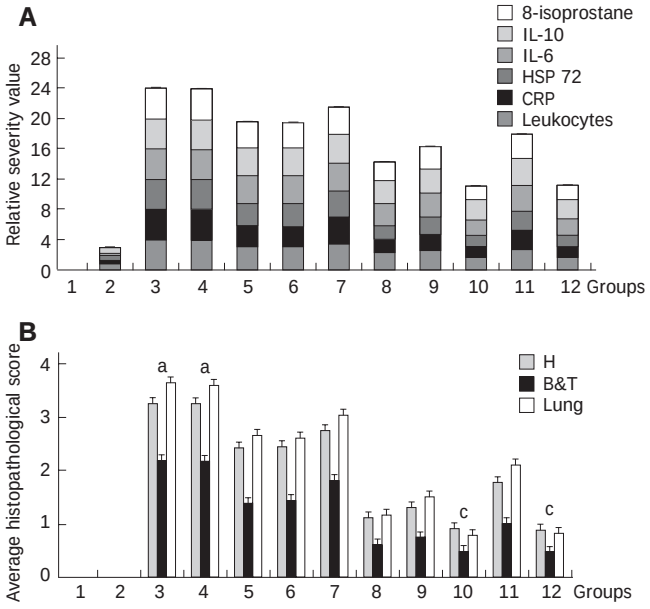

Figure 2 Relative severity value in blood and average histopathological score. **A**: Relative severity value in blood. To visualize the severity degree based on biochemical assays in blood, we arbitrarily established a single "relative severity value" of zero to group 1 (reference baseline value) and of four to group 3 (AP) per assay. To calculate the single "relative severity value" for the rest of the groups we used the following formula: "relative severity value" = [(X – reference baseline value) / (AP value – reference baseline value)] x 4. Introducing each mean value from table 2 (referred to in the above formula as X) we obtained six different single "relative severity values" for each group, whose contribution to the total "relative severity value" is shown in this graph. For those values in table 2 that were below the detection limit, the value assigned to X corresponded to that of the assay's sensitivity (IL-6: 112 pg/mL; IL-10: 12 pg/mL). Homogenous Groups are 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Results are shown as mean ± SD. **B**: Average histopathological score. To visualize the severity degree based on the score-associated to histopathological lesions, we averaged the means of the six parameters used in table 4 for each group. Homogenous groups are 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. There was a significant increase in the assessed parameters for Groups 3&4 that developed severe AP $^{a}P<0.03$ while groups 10& 12 exhibited a significant improvement $^{c}P<0.05$ Results shown as mean ± SD.

infiltrate, was significantly higher for homogenous groups (3-4) while homogenous Groups (10-12) that had been treated with relaxin before and after triggering acute pancreatitis presented lesser UMPO values. The Spearman Correlation Coefficient between MPO activity and leukocyte infiltrate was significant for the pancreatic cephalic segment ($R=0.943$, $P<0.01$); for the pancreatic splenic segment ($R=0.831$, $P<0.001$); and for the lung ($R=0.871$, $P<0.01$).

The increase in pancreatic wet weight due to edema was more significant for homogenous groups (5-6)-7-(3-4) than for homogenous groups (10-12).

Histopathological scores in pancreas and lung tissue

Homogenous groups (10-12) that developed a mild acute pancreatitis exhibited edema as the main histopathological feature and a lower value for pancreatic and lung leukocyte infiltrate that correlated with MPO activity. Homogenous groups (5-6)-7-(3-4) were characterized by high scores for leukocyte infiltrate that correlated with MPO activity, pancreatic hemorrhage as well as acinar and fat necrosis. Necrotic foci presented a spotted pattern with higher frequency in the cephalic segment. group 11 that received relaxin 4h after triggering acute pancreatitis were characterized by hemorrhage. The average of pancreatic histopathological scores presented a significant lower value for homogenous groups (10-12) when comparing them to the average values obtained for homogenous groups (5-6)-7-(3-4). These results are described in Table 4 and Figure 2B.

General AP histopathology Groups with severe AP presented parenchymal necrosis with neutrophil infiltrate, fat necrosis, congestive blood vessels in peri-pancreatic fat, and intense hemorrhage. The duodenum exhibited lysis in the mucosa, subserosal blood vessel congestion and severe neutrophil infiltrate but there was no evidence of possible leakage towards the cephalic segment. These groups exhibited alterations in the liver, lung, kidneys, spleen and suprarenal glands. The liver showed necrosis, hemorrhage, blood vessel congestion, neutrophil infiltrate and steatosis.

Table 4 Histopathological scores in pancreas and lung tissue (mean ± SD)

	Pancreatic Edema		Pancreatic Hemorrhage		Panc. Leuk. Infiltrate		Panc. Acinar Necrosis		Panc. Fat Necrosis		Lung Leuk
	H	B&T	H	B&T	H	B&T	H	B&T	H	B&T	Infiltrate
Groups											
1 Basal	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
2 Surgery control	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
3 AP	1.31±0.09	1.14±0.08	3.58±0.08	1.84±0.06	3.61±0.10	2.47±0.08	3.87±0.10	2.90±0.08	3.98±0.12	2.60±0.08	3.65±0.15
4 AP+PBS	1.33±0.07	1.12±0.10	3.55±0.10	1.82±0.07	3.64±0.09	2.46±0.11	3.83±0.09	2.86±0.12	3.95±0.10	2.66±0.11	3.61±0.10
5 Rlx+LN	1.01±0.06	0.73±0.10	2.46±0.10	1.08±0.06	2.93±0.14	1.53±0.07	2.96±0.10	2.03±0.05	2.84±0.11	1.57±0.08	2.66±0.10
6 Rlx+MIF	0.99±0.10	0.75±0.09	2.49±0.06	1.27±0.05	2.97±0.11	1.58±0.09	2.99±0.09	2.00±0.07	2.80±0.07	1.61±0.09	2.62±0.07
7 Rlx+LN+MIF	1.26±0.10	0.92±0.08	2.97±0.09	1.42±0.05	3.23±0.08	2.25±0.06	3.35±0.05	2.45±0.08	3.00±0.09	2.08±0.10	3.04±0.08
8 Rlx 1h pre	0.51±0.08	0.42±0.08	1.37±0.07	0.59±0.05	0.86±0.08	0.45±0.05	1.39±0.11	0.85±0.08	1.43±0.10	0.75±0.08	1.17±0.09
9 Rlx 1h post	0.62±0.07	0.50±0.08	1.50±0.08	0.71±0.08	1.03±0.03	0.62±0.06	1.64±0.07	1.01±0.06	1.71±0.08	0.98±0.05	1.52±0.10
10 Rlx 1h pre+4h post	0.42±0.07	0.37±0.06	1.26±0.10	0.43±0.07	0.72±0.07	0.37±0.09	0.92±0.09	0.59±0.05	1.19±0.09	0.63±0.09	0.80±0.08
11 Rlx 4h post	0.77±0.08	0.59±0.07	1.97±0.05	0.89±0.09	1.67±0.05	0.89±0.08	2.28±0.13	1.45±0.08	2.23±0.09	1.19±0.07	2.11±0.05
12 Rlx 12h&1h pre+4h post	0.45±0.05	0.36±0.05	1.24±0.08	0.45±0.04	0.70±0.05	0.38±0.06	0.90±0.05	0.60±0.05	1.20±0.05	0.61±0.07	0.83±0.06

Groups 3&4 that developed severe AP presented as main features leukocyte infiltrate, hemorrhage and acinar and fat necrosis and the histopathological scores were higher. Groups 10 & 12 that received relaxin 12 and/or 1h pre and 4h post surgery developed a mild AP and exhibited the lowest scores being the main feature edema. Homogenous groups for pancreatic edema, hemorrhage, acinar and fat necrosis and pancreatic and lung leukocyte infiltrate: :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

The lungs were highly compromised exhibiting congestion of big blood vessels and alveolar capillaries with neutrophil infiltrate; the bronchial wall had reactive lymphoid hyperplasia and aspirative hemorrhage. The main features observed in the kidneys were congestion of cortical and medullar blood vessels and glomerular capillaries jointly with tubular and medullar acute necrosis. Suprarenal glands were characterized by edema and partial lipid loss while the spleen presented edema.

Caspases activity (2-3-8-9)

We next examined if treatment of pancreatic injury was associated to the activation of caspases 2-3-8-9 in the pancreas as primary site of injury and in the lung and the liver as relevant affected distant organs. Homogenous groups (10-12) that developed a mild acute pancreatitis manifested a greater activation of caspases 2-3-8-9 suggesting that the activity of both the initiator caspase 8 and the executor caspase 3 together with caspases 9 and 2 involved in the apoptosome or intrinsic pathways are stimulated by relaxin treatment suggesting that apoptosis is more significant in these groups than in homogenous groups (5-6)-7-(3-4) that developed a severe necrotizing acute pancreatitis (Figure 3).

Death due to apoptosis or necrosis

The next step was to elucidate whether relaxin treatment favored death by apoptosis over necrosis. Blood lactate dehydrogenase (LDH) is a marker of cellular injury and death. The raise in blood LDH due to acute pancreatitis was ameliorated in homogenous groups (10-12) (Figure 4A). To evaluate if the undergoing death process in the pancreas as primary site of injury and in the lung and the liver as relevant affected distant organs corresponded to apoptosis or necrosis we measured ATP concentration (Figure 4B) and then calculated the % of loss of ATP (Figure 4C) given the fact that the death process involves ATP consumption being an 85% or more ATP depletion associated to necrosis. Also the ADP/ATP ratio (Figure 4D) helped to differentiate between these two death mechanisms as a higher increase is indicative of necrosis while a lower one points towards apoptosis.

ATP depletion for homogenous groups (5-6)-7-(3-4) associated to a higher % of ATP loss and ADP/ATP ratio correlated ($R=0.842$; $P<0.05$) with the higher frequency observed in necrotic foci and severity indicators for these groups together with the elevated LDH value suggesting that for groups undergoing severe acute pancreatitis necrosis was the main form of cell death.

Homogenous Groups (10-12) that manifested a mild acute pancreatitis evidenced lesser ATP depletion with a % of ATP loss lower than 40% together with a lower ADP/ATP ratio. These results correlated with lower values for LDH ($R=0.798$, $P<0.05$) while edema was the principal histopathological feature. This suggested that for these groups apoptosis was the main form of cell death resulting in a more controlled process with less pro-inflammatory stimulation ameliorating the aggressiveness of the pathology.

Increased degree of severity

For each variable with significant differences between the groups' mean values, the relationship of these results were compared to the reference baseline level established in group 1. We then integrated this information to rank the groups in order of "increased degree of severity" (Table 5) showing that the severe acute pancreatitis developed by homogenous groups (3-4) was significantly ameliorated for homogenous groups (10-12).

DISCUSSION

According to the severity degree exhibited by acute pancreatitis will be the complexity of the pathology involving other organs apart from the pancreas that will lead to metabolic, liver, kidney and lung alterations

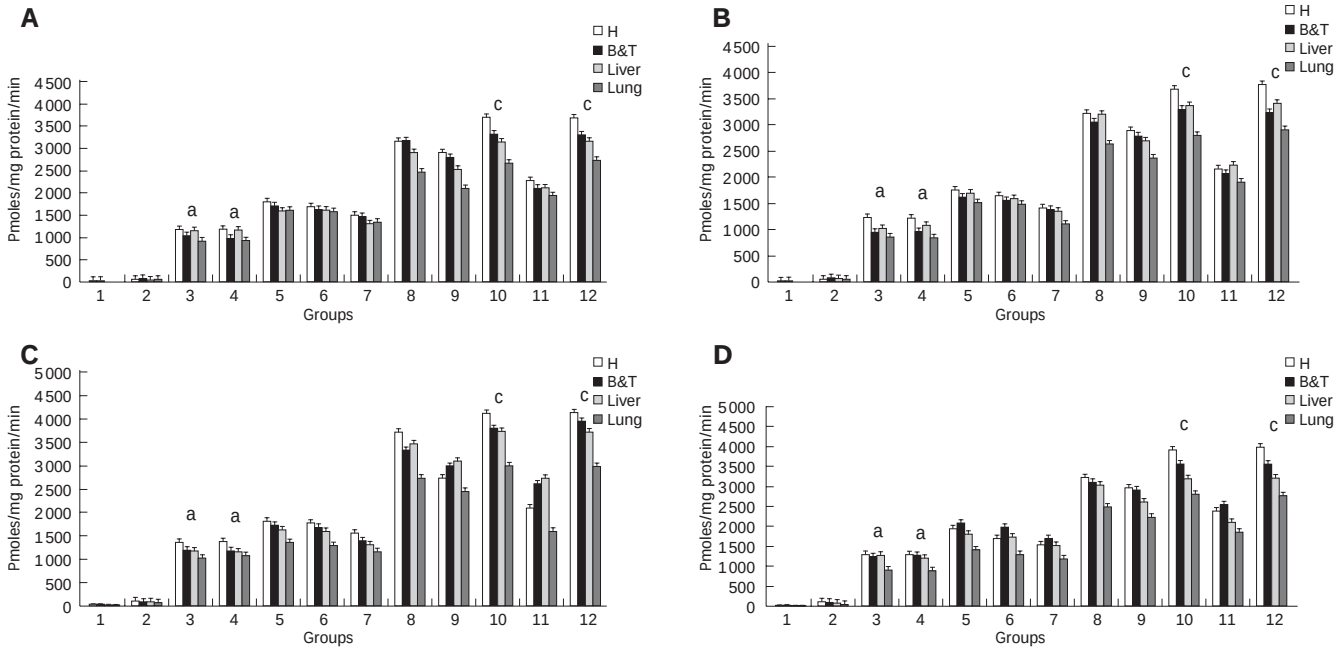

Figure 3 Caspase Activities. **A**: *Caspase 2 activity* was measured to evaluate the upstream activation of the intrinsic apoptotic pathway. Groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 2 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. **B**: *Caspase 3 Activity* was measured to evaluate the activation of the executor caspase 3. Groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 2 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. **C**: *Caspase 8 Activity* was measured to evaluate the activation of the initiator caspase 8. groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 8 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. Results shown as mean±SD. **D**: *Caspase 9 Activity* was measured to evaluate the activation of the intrinsic pathway .Groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 9 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. Results are shown as mean±SD.

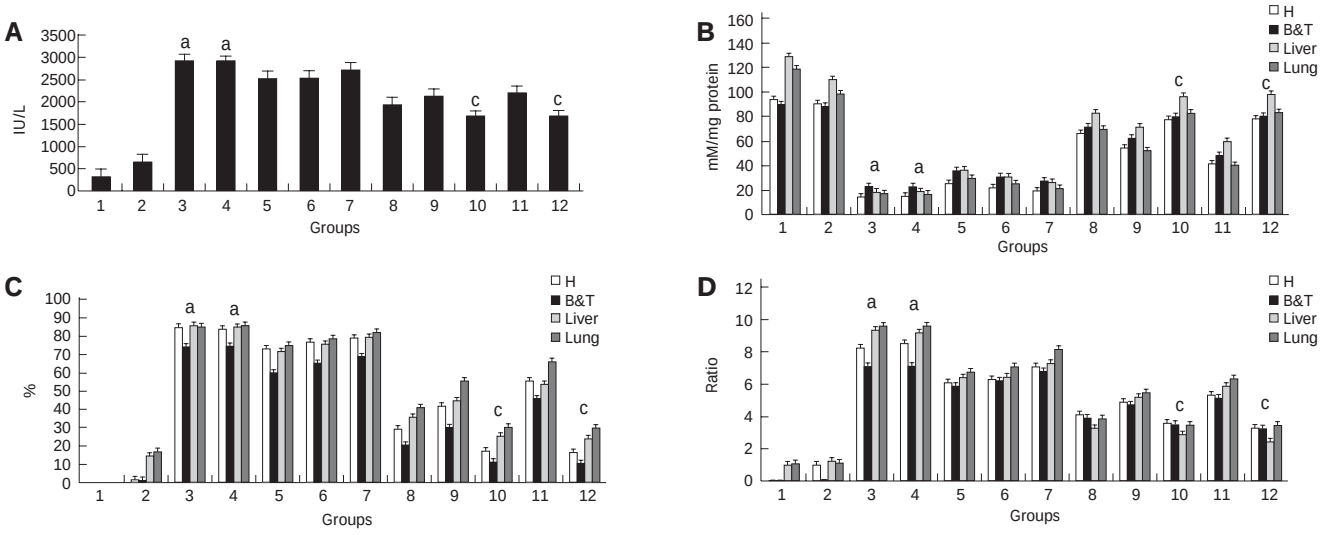

Figure 4 Apoptosis versus necrosis. **A**: Blood lactate dehydrogenase. As a marker of cellular injury and death significant high blood levels of LDH for groups 3&4 $^{a}P<0.005$ reflected the undergoing severe AP while groups 10&12 presented a significant reduction in the enzyme value $^{c}P<0.005$. Homogenous. Groups for LDH are:1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.005$. **B**-**D**: ATP concentration. The significant decrease in ATP concentration that surpassed the 80% loss for groups 3&4 $^{a}P<0.005$,is the hallmark of the necrotic process that is being developed while groups 10&12 that underwent apoptosis did not manifest such extreme values $^{c}P<0.005$. This expression in the nucleotide levels is translated into the ADP/ATP ratio that is significantly increased $^{a}P<0.01$ for groups 3&4 that underwent necrosis while groups 10&12 that underwent apoptosis had significantly lower values $^{c}P<0.05$. Homogenous Groups are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.005$. Results are shown as mean±SD.

reflected in the histopathology and numerous biochemical parameters that require to be assessed in order to arrive to a proper classification of the disease.

It has been reported that relaxin can stimulate the nitric oxide pathway or the glucocorticoid receptor[7,14]. In our work we have evaluated the combined vasorelaxing and anti-inflammatory properties of relaxin on the evolution of acute pancreatitis, our results show that relaxin plays a highly beneficial role when administered within the first hour of the initiation of the noxa being this even more pronounced when the drug was given 1h pre and 4h post triggering acute pancreatitis, this would be particularly relevant for the prevention of post-ERCP pancreatitis providing basis for future studies where combined drug

Table 5 Order of groups according to the increase in the severity degree

GROUP NR.	GROUP DESCRIPTION
1	Basal Reference Control
2	Surgery Control
12	Relaxin administered 12h & 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
10	Relaxin administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
8	Relaxin administered 1h pre-BPDOE-CDLs
9	Relaxin administeres 1h post-BPDOE-CDLs
11	Relaxin administered 4h post-BPDOE-CDLs
5	Relaxin and L-NAME administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
6	Relaxin and Mifepristone administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
7	Relaxin, L-NAME & Mifepristone administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
3	BPDOE-CDLs AP model
4	Vehicle (PBS) control

The BPDOE-CDLs model induces a severe AP (group 3).Prophylactic treatment with relaxin, as shown for groups10&12, markedly reduces the severity of the pathology, being still useful although in a lesser degree even after AP was triggered.

treatments can be considered. When relaxin was received 4h after unchaining acute pancreatitis although still beneficial the efficiency of the treatment was reduced the reason probably being that additional supplemental therapies are required at this stage in order to restore the already altered hemodynamic and metabolic balance. The positive effect exerted by relaxin was significantly diminished by L-NAME and by mifepristone indicating that the nitric oxide pathway and glucocorticoid receptor stimulation are involved. The association of both inhibitors produced an even stronger reduction in the effect induced by relaxin however this group still presented less severe results than those observed for non-treated acute pancreatitis suggesting that there may be other pathways implicated.

While *apoptosis* is a programmed and controlled mechanism of cell death that is associated to reduced inflammation, *necrosis*, caused by irreparable cell injury causing membrane alterations that favor the release of enzymes that are responsible for degrading cellular structures and thus activating the inflammatory response[25]. Pancreatic hemorrhage and necrosis characterize severe acute pancreatitis being the consequence of dysfunctional tissue irrigation due to an initial hypoxia leading to an ischemia-reperfusion process due to alternative vasoconstriction followed by vasodilatation. These microcirculatory disturbances are linked to neutrophil activation and inflammation together with cellular stress that will convert cell death by apoptosis to necrosis, downregulating these factors by preserving pancreatic microvascular blood flow to ensure adequate tissue oxygenation and inhibiting pro-inflammatory mediators reduces the risk of necrotizing pancreatitis by favoring cell death by apoptosis over necrosis.

The fact that the severity of acute pancreatitis is inversely related to the degree of apoptosis suggests that this type of cell death may be a beneficial response to acinar cell injury particularly in acute pancreatitis. Clearly, the degree of inflammation and, therefore, the severity of pancreatitis are less in situations associated with acinar cell apoptosis than under conditions associated with acinar cell necrosis. The group of Steer[25] emphasizes then the notion that medications or other interventions that favor the development of apoptosis may minimize the severity of pancreatitis, and they could, therefore be of substantial clinical value. Together with Jones and Gores[26] they remark the concept that pharmacological induction of pancreatic apoptosis during the early stages of acute pancreatitis provides a relevant therapeutic strategy for the treatment of this disease.

The high density of neurons embedded in the pancreatic cephalic segment associated to the intimate relation between the peri-vaterian duodenum and the pancreas linked by the gastroduodenal plexus[27-29] emphasizes the relevance of the rich population of receptors in the peri-vaterian duodenum that when activated by irritative stimuli such as endoscopic maneuvers will unchain duodenopancreatic and duodenogastric autonomic-arc-reflexes. The BPDOE-CDLs model mimics this situation in which severe acute pancreatitis results from a sympathetic neurogenic overstimlaution that causes adrenergic hyper-reactivity responsible for the initial vasoconstriction and the subsequent ischemia/reperfusion injury. We propose that relaxin would, in first instance, counteract vasoconstriction arising from sympathetic-adrenergic stimulation by inducing vasodilatation through the nitric oxide pathway and exert anti-inflammatory properties by glucocorticoid receptor stimulation while favoring apoptosis over necrosis.

Severe acute pancreatitis induced by the BPDOE-CDLs model manifested kidney failure translated by the significant increase in urea and creatinine in blood associated to acute tubular necrosis; metabolic and liver alterations. The raise in severity factors indicating an intense inflammatory response defined by the increase in CRP and leukocyte count that correlated with neutrophil infiltrate in pancreatic and lung tissue associated to high activity values for MPO in these tissues suggested a SIRS in which the activation of neutrophils converted cellular death by apoptosis to necrosis as suggested by the high necrosis score in pancreatic tissue and the elevation of blood LDH describing organ injury. For this group caspases 2-3-8 and 9 activities were significantly reduced when comparing them to treated homogenous groups

(10-12). The dramatic depletion in ATP concentration, correlated with a loss of ATP that surpassed the 80 % thus inhibiting the initiation of the apoptotic pathway, and the increase in ADP/ATP ratio would be indicating a strong necrotic mechanism associated to a pro-inflammatory process as corroborated by the histopathology.

Groups that were treated with relaxin showed an improvement in blood metabolic parameters with reduced values for leukocyte count and CRP together with lower leukocyte infiltrate and MPO activity in pancreatic and lung tissue pointing to a milder inflammatory response. The increase in the activity of caspases 2-3-8 and 9 manifesting that both the intrinsic and the effector apoptotic pathways are activated is associated to a less drastic depletion of ATP that would not interfere with apoptosis and thus exhibit a reduced inflammatory reaction. Treatment with relaxin 4h after unchaining acute pancreatitis was beneficial however the best results were observed when relaxin was given within the first hour being optimal when administered 1h before and 4h after triggering acute pancreatitis as quite probably in this circumstance it could preserve microcirculation ensuring tissue oxygenation while inhibiting the expression of pro-inflammatory mediators associated with pro-apoptotic molecules, situation that is kept-up by the second dose, these results are relevant for the prevention of post-ERCP acute pancreatitis. There was no significant difference between homogenous groups 10 and 12 implying that starting treatment 12h or 1h prior to triggering acute pancreatitis exerts the same effect.

To evaluate the pathways involved in treatment with relaxin we associated the administration of relaxin 1h pre and 4h post BPDOE-CDLs procedure with L-NAME (NOS inhibitor) and/or mifepristone (glucocorticoid recptor antagonist). The inhibition of either the nitric oxide pathway or glucocorticoid receptor stimulation significantly reduced the positive effects described previously for homogenous groups (10-12). Surprisingly for group 7 when we associated both inhibitors the severity that was developed although greater than for homogenous groups (6-7) was still less than that displayed by homogenous groups (3-4) suggesting that there is another pathway involved in the treatment with relaxin.

We therefore conclude that relaxin plays a beneficial role in the evolution of acute pancreatitis triggered by the BPDOE-CDLs model as it induces an anti-inflammatory response via glucocorticoid receptor stimulation while favoring apoptosis over necrosis; simultaneously it helps to preserve pancreatic microcirculation by the nitric oxide pathway ensuring an appropriate blood flow and tissue oxygenation thus avoiding ischemia and the consequent ischemia/reperfusion injury while it reduces the expression and activation of pro-inflammatory factors and decreases neutrophil infiltration of the pancreas.

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BASIC RESEARCH

Cis-hydroxyproline-induced inhibition of pancreatic cancer cell growth is mediated by endoplasmic reticulum stress

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Abstract

AIM: To investigate the biological effects of cis-hydroxyproline (CHP) on the rat pancreatic carcinoma cell line DSL6A, and to examine the underlying molecular mechanisms.

METHODS: The effect of CHP on DSL6A cell proliferation was assessed by using BrdU incorporation. The expression of focal adhesion kinase (FAK) was characterized by Western blotting and immunofluorescence. Induction of endoplasmic reticulum (ER) stress was investigated by using RT-PCR and Western blotting for the glucose-related protein-78 (GRP78) and growth arrest and DNA inducible gene (GADD153). Cell viability was determined through measuring the metabolic activity based on the reduction potential of DSL6A cells. Apoptosis was analyzed by detection of caspase-3 activation and cleavage of poly(ADP-ribose) polymerase (PARP) as well as DNA laddering.

RESULTS: In addition to inhibition of proliferation, incubation with CHP induced proteolytic cleavage of FAK and a delocalisation of the enzyme from focal adhesions, followed by a loss of cell adherence. Simultaneously, we could show an increased expression of GRP78 and GADD153, indicating a CHP-mediated activation of the ER stress cascade in the DSL6A cell line. Prolonged incubation of DSL6A cells with CHP finally resulted in apoptotic cell death. Beside L-proline, the inhibition of intracellular proteolysis by addition of a broad spectrum protease inhibitor could abolish the effects of CHP on cellular functions and the molecular processes. In contrast, impeding the activity of apoptosis-executing caspases had no influence on CHP-mediated cell damage.

CONCLUSION: Our data suggest that the initiation of ER stress machinery by CHP leads to an activation of intracellular proteolytic processes, including caspase-independent FAK degradation, resulting in damaging pancreatic carcinoma cells.

Key words: Cis-hydroxyproline; Pancreatic cancer cell; endoplasmic reticulum stress; FAK; Caspase-3

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INTRODUCTION

The prognosis of pancreatic carcinoma is uniformly fatal. Conventional cytostatic treatments for inoperable pancreatic cancer make little impact on the median survival. Therefore, the development of novel therapeutic concepts that activate proliferation-independent cell death are needed[1]. Cell damage by targeting protein biosynthesis may be a promising strategy for cancer therapy[2,3]. Previous studies have shown that cis-4-hydroxy-L-proline (CHP) as an analogue of L-proline inhibits the collagen synthesis and its extracellular deposition[4,5]. Furthermore, there are reports describing inhibitory effects of CHP on proliferation, adhesion, and migration of various cell types like fibroblasts, epithelial cells and tumor cells[5-8]. These data pointed to additional and/or other proteins whose synthesis might be affected by incorporation of the incorrect amino acid leading to disturbances of cellular mechanisms.

Based on the CHP-induced inhibition of cell adhesion, we hypothesized that the focal adhesion kinase (FAK) may be involved in the CHP-induced signal transduction. FAK is a 125 ku non-receptor tyrosin kinase that mediates different cellular responses to adhesion after clustering of transmembrane integrins at contact sites between the cell and the extracellular matrix (ECM) termed focal adhesions[9]. Frisch *et al*[10] have shown that constitutively activated FAK conferred protection against anoikis of epithelial cells. Consequently, the inhibition

of FAK resulted in apoptosis of fibroblasts as well as in detachment and cell death of human breast tumor cells, suggesting a pivotal role of FAK in the transmission of anti-apoptotic signals[11,12].

The incorporation of amino acid analogues into newly synthesized proteins has been shown to interfere with the correct protein folding leading to an accumulation of misfolded proteins in the endoplasmic reticulum (ER)[13]. The ER has emerged as an organelle that is exquisitely sensitive to alterations in homeostasis that initiates a diversity of molecular defence mechanisms referred to as 'ER stress'[14]. The complex ER stress response to a variety of different stimuli results eventually in adaptation for survival or induction of apoptosis[14]. Mechanisms activated upon accumulation of unfolded protein in the ER should ensure that only properly folded proteins abandon the ER lumen[15]. This quality control system includes the unfolded protein response (UPR) which is characterized by an up-regulation of chaperons, such as the glucose-regulated protein-78 (GRP)78, as well as by an induction of transcription factors like the pro-apoptotic growth arrest and DNA gene product (GADD)153[16].

The aim of this study was to characterize the biological effects of CHP on the rat pancreatic tumor cell line DSL6A. Addressing the question of the underlying molecular mechanisms, we investigated the participation of ER stress and FAK in the CHP effect on cell proliferation, adhesion, survival and apoptosis.

MATERIALS AND METHODS

Reagents

Dulbecco's minimal essential medium (DMEM) and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). Iscove's modified Dulbecco medium (IMDM) was purchased from PAA Laboratories (Cölbe, Germany). Nonessential amino acids, penicillin, streptomycin, trypsin, and all reagents for reverse transcription [Superscript RT, oligo(dT)$_{12-18}$, dNTP] were purchased from Invitrogen (Karlsruhe, Germany). Cis-4-hydroxy-L-proline were from Riemser Arzneimittel AG (Greifswald, Germany). Colorimetric 5-bromo-2'-deoxyuridine (BrdU)-labelling cell proliferation ELISA and apoptotic DNA ladder kit were obtained from Roche (Mannheim, Germany). CellTiter AQ$_{ueous}$ kit from Promega (Mannheim, Germany), RNeasy Mini RNA extraction kit and Taq polymerase from Qiagen (Hilden, Germany) were used. Primers for PCR were generated by using NCBI GenBank as the source for any sequences and were synthesized by BioTez (Berlin, Germany). Mouse monoclonal anti-FAK$^{(N354-534)}$ was purchased from Transduction, mouse anti-GRP78 from BD Biosciences, anti-β-actin from Sigma (Taufkirchen, Germany), anti-cleaved caspase-3 from anti-poly-(ADP ribose) polymerase (PARP), anti-cyclinD1 from Santa Cruz. Secondary horseradish peroxidase-labelled anti-mouse or anti-rabbit Ig antibodies, nitrocellulose membranes, ECL plus kit and hyperfilm ECL were purchased from Amersham (Freiburg, Germany). Alexa Fluor® 488 goat anti-mouse IgG was purchased from MoBiTec (Göttingen, Germany). Caspase-3 inhibitor Z-DEVD-FMK, general caspase inhibitor Z-VAD-FMK and the protease arrest reagent were purchased from Calbiochem (Schwalbach, Germany).

Cell culture

The rat pancreas carcinoma cell line DSL6A was cultured in DMEM supplemented with 100 mL/L FCS, 100 U/mL penicillin and 100 μg/mL streptomycin or in IMDM containing non-essential amino acids in addition to 100 mL/L FCS and antibiotics.

In contrast to IMDM, DMEM did not contain L-proline. To prevent cell adhesion, culture plates were coated with 20 g/L agarose as previously described[17].

Cell proliferation and cell survival

Cell proliferation was assessed using the colorimetric 5-bromo-2´-deoxyuridine (BrdU) DNA incorporation assay. Cells suspended in DMEM supplemented with 100 mL/L FCS were seeded into 96-well plates ($1x10^4$ cells/well). After becoming adherent, the cells were treated for 24 h and 48 h with different CHP concentrations dissolved in serum-free DMEM. BrdU labelling was performed during a further 20-h incubation. The BrdU incorporation was quantified using the ELISA kit according to the manufacturer's instructions[18].

For the detection of alive cells, the metabolic activity was measured by a colorimetric method based on the conversion of the tetrazolium salt MTS into formazan using the CellTiter AQ$_{ueous}$ kit according to the manufacturer's instructions. Cell cultivation and incubation was performed as described for BrdU labelling.

Apoptosis

Apoptosis was detected by the cleaved caspase-3 as well as the degradation of poly(ADP-ribose) polymerase (PARP) using the immunoblot technique. To show DNA laddering, cells were seeded into 12-well plates. Adherent cells were cultured in serum-free DMEM for 24 h and 48 h with or without 10 mmol/L CHP. At the end of the incubation, cells were harvested and DNA was extracted using the apoptotic DNA-Ladder kit according to the manufacturer' s instructions. Isolated DNA was electrophoretically separated on 15 g/L agarose gel containing ethidium bromide. The ethidium bromide fluorescence was visualised using an electronic camera.

RT-PCR

Total RNA was isolated using the RNeasy extraction kit. Three microgram of total RNA was reversed transcribed into cDNA as previously described[19]. Quantification of mRNA expression was preformed by using competitive PCR for the housekeeping gene β-actin and co-amplification with a defined concentration of a synthetic DNA fragment as internal standard[19]. On this basis, various cDNA samples were adjusted to equal input concentrations and electrophoretically separated on 18 g/L agarose gel containing ethidium bromide. Visualisation and measurement of resulting bands were performed with an electronic camera analysing the data with the EASY program (Herolab, Wiesloch, Germany). The primers for RT-PCR were as follows: for GRP78, GACATTTGCCCCAGAAGAAA (sense) and

ATGACCCGCTGATCAAAGTC (antisense), product size 375 bp; for GADD153, AGCTGAGTCTCTGCCTTTCG (sense) and TGTGGTCTC TACCTCCCTGG (antisense), product size 456 bp[20].

Western blot analysis

Cells were seeded into 60-mm dishes and grown to confluence. Protein extracts of DSL6A cells were prepared as previously described[18]. Proteins were electrophoretically separated on an 80 g/L SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. Blots were incubated with the respective primary antibody for 2 h at room temperature. For visualization of the antibody binding, filters were exposed to a horseradish peroxidase (POD)-labelled anti-rabbit or anti-mouse Ig antibody and developed using the ECL Plus kit. The following primary antibody dilutions were used: FAK 1:1000, GRP78 1:500, cleaved caspase-3 1:500, PARP 1:1000, β-actin 1:1000. For re-probing with additional antibodies, membranes were stripped of bound antibodies by treatment with stripping buffer containing 62.5 mmol/L Tris (pH 6.7), 2 g/L SDS, 7 mL/L 2-mercaptoethanol for 30 min at 50 ℃.

Immunofluorescence

Cells were cultivated on glass cover slips as previously described[18]. For immunofluorescence staining, cells were fixed with ice-cold methanol, followed by incubation with a mouse monoclonal antibody (mAB) detecting FAK. Binding of the specific mAB was determined by Alexa Fluor anti-mouse IgG and visualized using the Confocal LASER scanning microscope (Leica TCS SP2 AOBS).

Statistical analysis

Results were expressed as mean ± SE. Data were analyzed using Wilcoxon's rank sum test. $P < 0.05$ was considered statistically significant.

RESULTS

CHP-induced morphological appearance of DSL6A cells

The incubation with 10 mmol/L CHP induced rounding up of adherently growing DSL6A cells resulting after 24 h in a complete detachment (Figure 1B). The CHP antagonist L-proline circumvented this CHP-induced loss of adherence (Figure 1C). Moreover, cells which had been rounded up through CHP treatment, spread out again after L-proline addition, forming finally a normal monolayer (Figure 1D).

Effects of CHP on cell proliferation and viability

Figure 2A shows the influence of CHP on the S phase of the cell cycle as measured by BrdU incorporation into the DNA of DSL6A cells. We observed CHP significantly inhibited the proliferation in a time- and dose-dependent manner. The determination of metabolically active DSL6A cells (Figure 2B) revealed a discrepancy between the efficiency of CHP in regard to cell proliferation and viability. While the BrdU incorporation after treatment with 10 mmol/L CHP for 24 h averaged only 20% of the controls (Figure 2A), cell viability was more than 50% (Figure 2B). The results obtained by BrdU incorporation

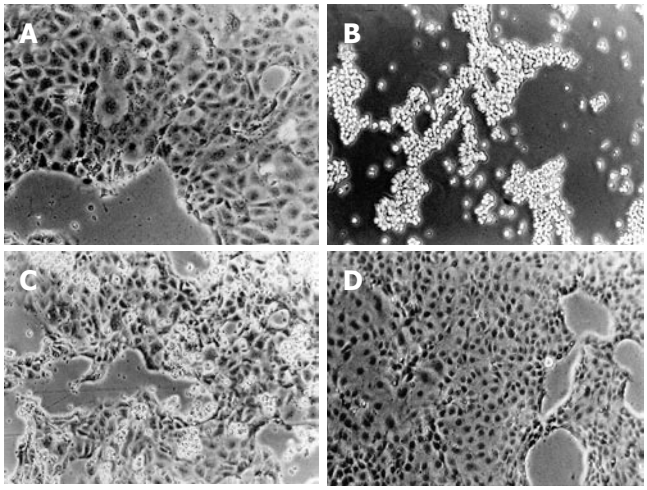


Figure 1 Effect of CHP on morphological appearance of DSL6A cell (Phase contrast microscopy, original magnification x200). A: Adherently growing controls; B: Incubation with 10 mmol/L CHP for 24 h caused rounding, followed by detachment of DSL cells; C: Addition of 20 mmol/L L-proline abolished the effects of CHP; D: CHP-treated cells cultured for further 48 h with 20 mmol/L L-proline, resulting in the re-adherence of the cells.

have been reflected on the molecular level analysing cyclin D1, a molecule substantially involved in the cell cycle progression[21]. As shown by immunoblotting, CHP triggered a decline of the cyclin D1 protein (Figure 2C). The CHP-caused growth inhibition could be prevented through addition of L-proline, indicating a competitive replacement of CHP by the correct amino acid (Figures 2A and 2B).

To examine whether the impaired cell functions could be restored, L-proline was added to the CHP-containing culture medium 24 h after beginning of the incubation with CHP. Cells were allowed to grow for further 24 h, followed by the measurement of BrdU incorporation. As shown in Figure 2D, the addition of L-proline induced a re-increase in BrdU incorporation.

In order to exclude the possibility that the restoring of proliferation was due to residual intact cells, we measured the BrdU uptake of cells remaining in the culture plates after removal of cells detached by CHP treatment. The BrdU incorporation was not significantly higher than immediately after CHP incubation (data not shown). These results suggested that CHP-induced DSL6A cell detachment is a reversible process.

Influence of CHP on FAK

The strong inhibition of cell adherence by CHP pointed to an involvement of the focal adhesion kinase (FAK) in the CHP-mediated molecular mechanisms[10]. Analysis of FAK using the Western blot technique revealed the expected 125 ku protein (Figure 3A). Treatment of the cells with CHP caused a time-dependent decline of the FAK signal and a simultaneous appearance of fragments of about 80 ku, suggesting a proteolytic degradation of the FAK molecule leading to a complete fragmentation during 48 h (Figure 3A).

Based on the hypothesis that CHP brought about FAK inactivation by proteolytic cleavage of the enzyme, we tested the influence of a broad spectrum protease inhibi-

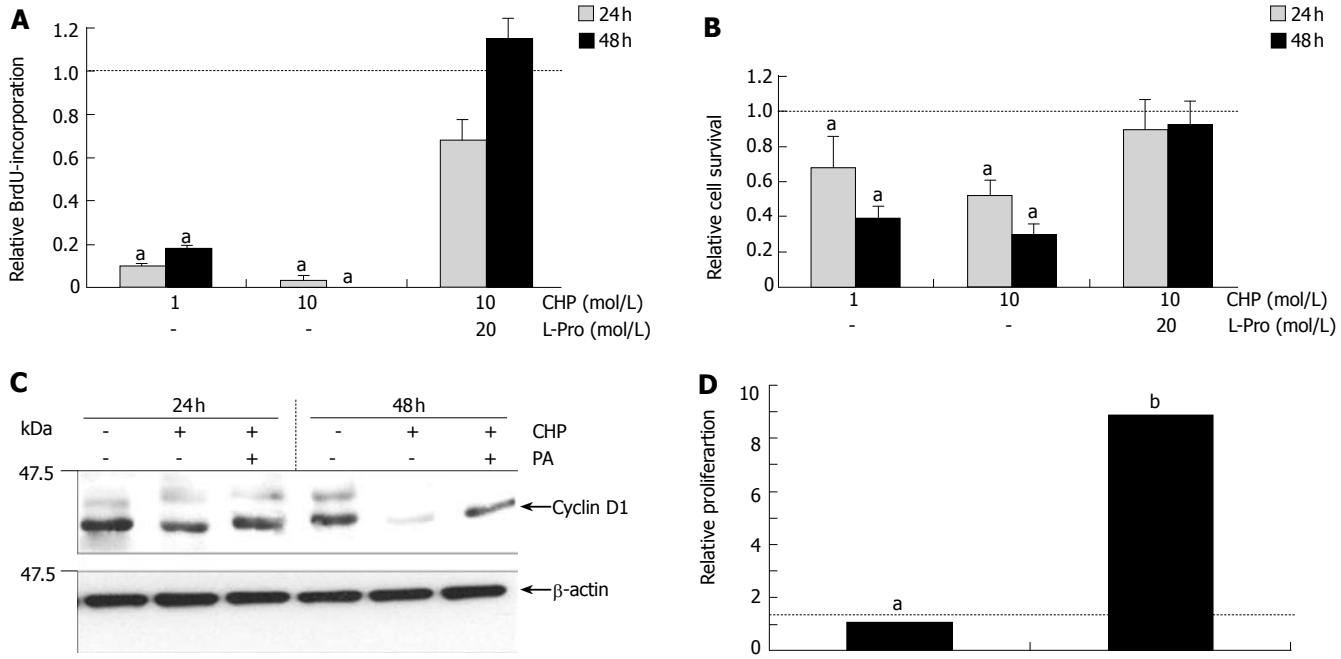
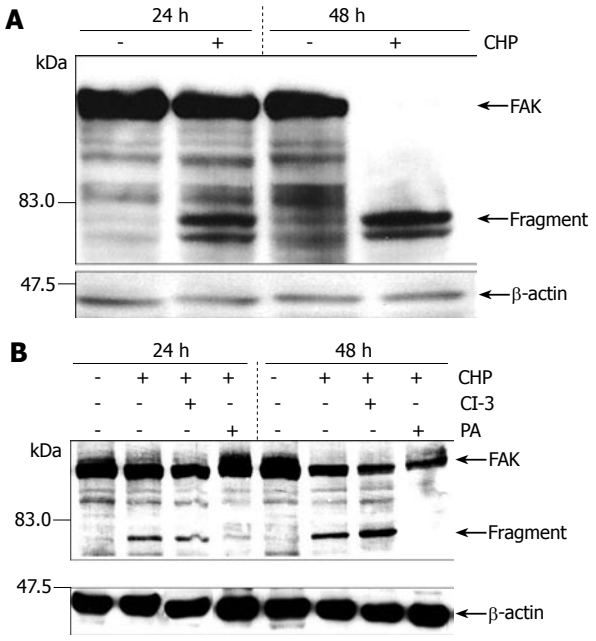


Figure 2 Inhibition of cell proliferation and survival by CHP. A: After the addition of BrdU, cells were cultured for further 20 h, followed by quantification of BrdU incorporation; B: Four hours after the addition of CellTiter AQueous solution, conversation of the MTT salt was measured. CHP-caused decrease of proliferation (A) and metabolically active cells (B) was prevented by the addition of L-proline. Results are expressed as mean±SE (n = 6, $^{a}P \leqslant 0.05$). C: Immunoblotting of total cell extracts from DSL6A cells using an anti-cyclin D1 antibody. Results are representative for three independent experiments. D: DSL6A cells were treated with 10 mmol/l CHP for 24 h before culture medium was (a) replaced by DMEM without CHP or (b) 20 mmol/L L-proline was added to the CHP-containing medium.



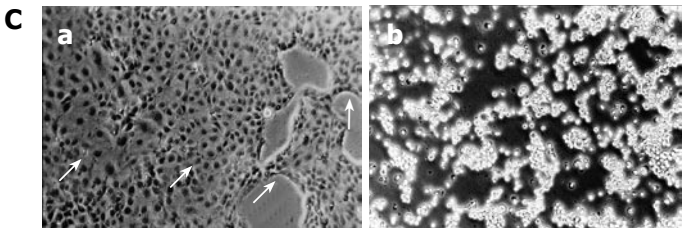


Figure 3 FAK fragmentations in DSL6A cells induced by CHP. A Immunoblotting of protein extracted from DSL6A cells after being incubated for 16 h and 48 h with or without 10 mmol/L CHP. CHP induced a time-dependent degradation of the 125 ku FAK protein, resulting in fragments of about 80 ku. B Simultaneous addition of the Protease Arrest Reagent (PI) to the CHP-treated cells attenuated fragmentation of FAK; in contrast, the caspase-3 inhibitor z-DEVD-fmk (CI-3, 100 μmol/L) was not able to abolish the CHP-induced FAK degradation. To show equal protein loading, the membranes were stripped and re-probed using an anti-β-actin specific antibody. C Effect of CHP on morphological appearance of DSL6A cells; (a) simultaneous addition of 10 mmol/L CHP and a broad spectrum protease inhibitor (PI, Protease Arrest Reagent) abolished the CHP-induced loss of adherence; (b) In contrast to the broad spectrum protease inhibitor, the caspase-3 inhibitor z-DEVD-fmk (CI-3, 100 μmol/L) had no effect on the CHP-induced loss of adherence (Phase contrast microscopy, original magnification x 200).

tor [protease arrest (PA) reagent]. The repression of the intracellular proteolytic activity was able to abolish the CHP-induced FAK degradation (Figure 3B). In addition, pre-incubation of DSL6A cell cultures with the protease inhibitor prevented the CHP-caused cell detachment (Figure 3Ca). Moreover, the cyclin D reduction in DSL6A cells treated for 48 h with CHP was attenuated by the PA reagent (Figure 2C)

In contrast, the inhibition of caspase-3 activity by Z-DEVD-FMK (CI-3) had neither an effect on CHP-mediated FAK degradation (Figure 3B) nor on cell detachment (Figure 3Cb). In addition, treatment with Z-VAD-FMK, a general caspase inhibitor including caspase-3, 7, 8, 10, could not abolish CHP-caused influences on DSL6A cells (data not shown).

Since FAK is localized in focal adhesions while the cell is in contact with extracellular matrix, we, using immunofluorescence staining, analysed the influence of CHP on the intracellular assembly of the enzyme. Results were visualized by laser scanning microscopy (Figure 4). We clearly observed the focal adhesions in adherently growing control cells (Figure 4A), whereas the CHP-caused cell

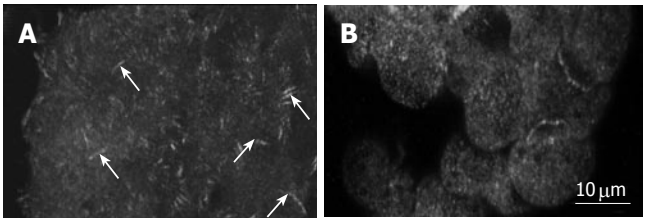


Figure 4 Immunocytochemical localisation of FAK. DSL6A cells grown on glass cover slips were cultured for 24 h without (A) or with 10 mmol/L CHP (B). Cell staining was performed by incubation with a mouse anti-FAK specific antibody. Binding of the primary antibody was detected by an AlexaFluorTM-labelled anti-mouse antibody. Arrows: focal adhesions; Bar: 10 μm.

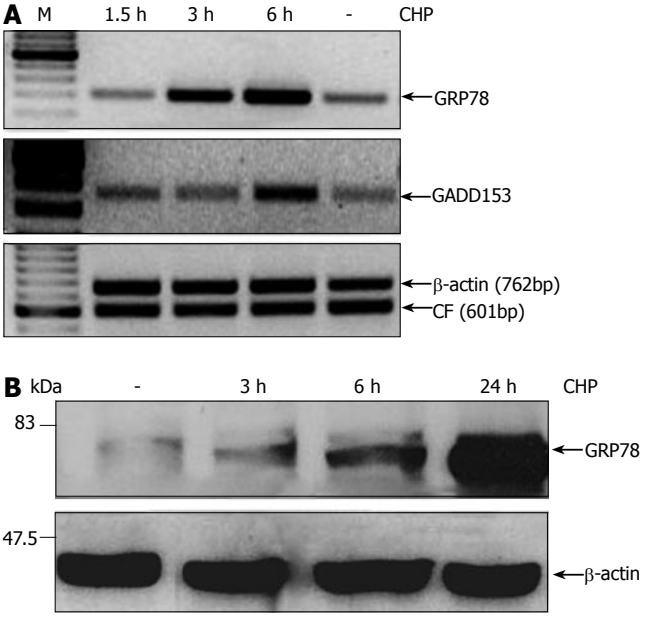


Figure 5 Influence of CHP on expression of GRP78 and GADD153. A: RT-PCR; M = 100-bp molecular weight marker; B: Western blot.

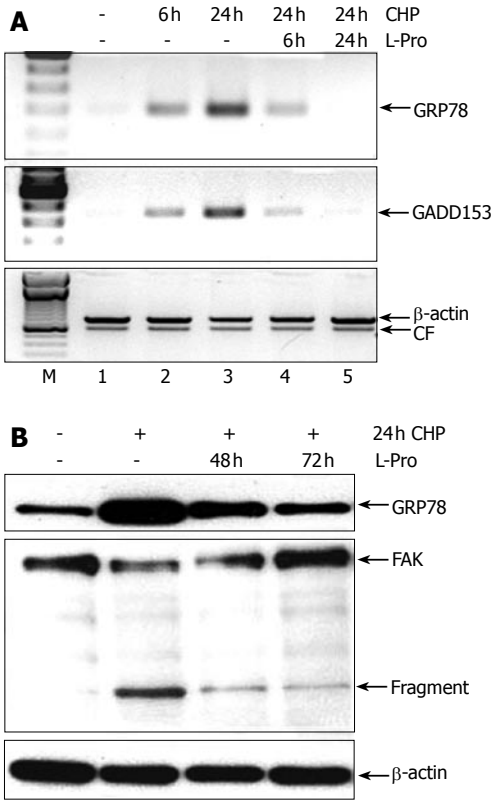


Figure 6 Reversion of CHP-mediated molecular effects by L-proline. A: RT-PCR analysis of mRNA expressions of GRP78 and GADD153. Lane 1: DSL6A cells cultured without 10 mmol/L CHP for 6 h; lane 2: DSL6A cells cultured with 10 mmol/L CHP for 6 h; lane 3: DSL6A cells cultured with 10 mmol/L CHP for 24 h; lane 4: DSL6A cells treated for 24 h with CHP after the addition of 20 mol/L L-proline for further 6 h; lane 5: DSL6A cells treated for 24 h with CHP after the addition of 20 mol/L L-proline for further 24 h. B: Western blot analysis of GRP78 and FAK protein. Results are representative of at least three independent experiments.

rounding resulted in a loss of focal adhesions and a diffuse distribution of the FAK protein (Figure 4B).

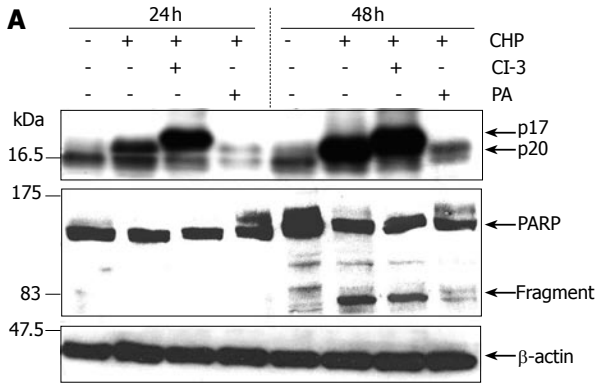
Influence on the expression of ER stress markers GRP78 and GADD153

CHP was earlier described as an inhibitor of normal collagen folding[22], a condition that inevitably affects the homeostasis of the endoplasmic reticulum (ER) referred to as ER stress[15]. In order to test whether CHP effects are mediated by ER stress, we analyzed the expression of GRP78 and GADD153 proteins whose up-regulation was defined as a component of the unfolded protein response (UPR)[18,15]. We found that CHP induced the mRNA expression of the both ER stress markers (Figure 5A). GRP78 transcript levels were found to be increased 3 h after the addition of CHP, while the up-regulation of GADD153 was detected only after 6 h. The CHP-induced GRP78 expression was confirmed on protein level by immunoblotting. A modest increase of the UPR component could be detected 3 h after the addition of CHP. The GRP78 protein levels in the cells increased during prolonged incubation with CHP reaching a maximum after 24 h (Figure 5B).

To test whether the L-proline-caused restoration of cell functions (Figure 2) was reflected on the molecular level, we investigated the time course of the expression of GRP78 and GADD157 during incubation with L-proline in CHP-treated cells. Six hours after the addition of L-proline, the transcript levels of both UPR components were reduced, reaching normal values during 24 h (Figure 6A). In contrast, the decrease of GRP78 protein levels could be detected only after 48 h (Figure 6B). Furthermore, L-proline-induced reversion of the CHP-mediated impairment of cell functions was accompanied by a time-dependent reconstitution of the FAK molecule associated with a reduction of the protein fragments (Figure 6B).

Impact of CHP on apoptosis

The reduction in cell viability pointed to the induction of apoptosis by CHP. Using the Western blot technique, we analyzed the processing of caspase-3 and poly-(ADP ribose) polymerase (PARP) to verify programmed cell death. The activation of apoptosis-executing caspase-3 occurs in a two-step proteolytic process. Initially, the procaspase-3 is cleaved into the subunits p12 and p20, followed by an autocatalytic fragmentation of the latter one, resulting in the fully active p17 enzyme[23]. Treatment of



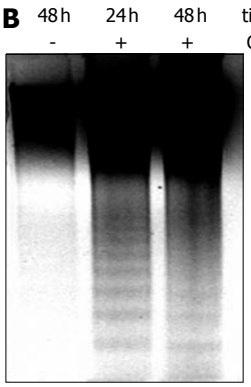
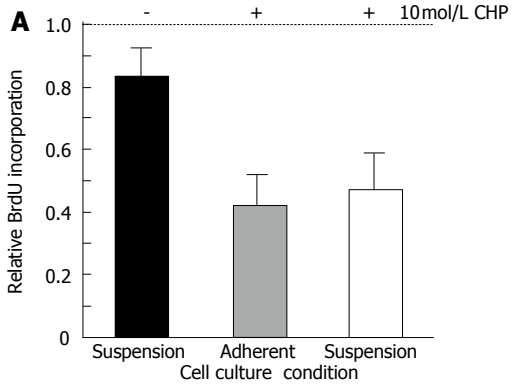


Figure 7 Induction of apoptosis by CHP. A: Immunoblotting using specific antibodies for cleaved caspase-3 or poly (ADP) ribose polymerase (PARP). B: DNA laddering induced by CHP. Results are representative of at least three independent experiments.



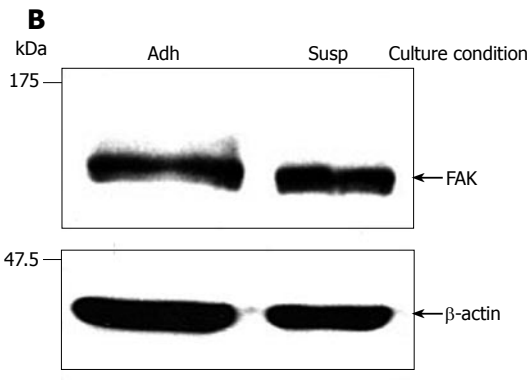


Figure 8 Survival of DSL6A cells cultured in suspension. A: Assessment of cell viability using the CellTiter AQueous assay. Adherent cells (adh.) and cells in suspension (susp.) were incubated for 24 h with or without 10 mmol/L CHP. Results are expressed as a percentage of living cells with regard to adherently growing, untreated control. Data are expressed as mean±SE (n=6). B: Western blot analysis of FAK protein levels.

DSL6A cells with CHP caused a time-dependent activation of caspase-3 represented by the p17 molecule (Figure 7A). Similarly, the cleaved PARP protein of 85 ku occurred during CHP treatment (Figure 7A). Intriguingly, the CHP-induced apoptotic DSL6A cell death, determined by the activation of caspase-3 and PARP, could be prevented by pre-treatment of the cells with the broad spectrum protease inhibitor PA (Figure 7A). These results together with the prevention of CHP-induced FAK degradation (Figure 3B) as well as cyclin D1 reduction (Figure 2C) by inhibition of intracellular proteolysis indicate a superior role of proteases in the CHP-mediated effects.

The caspase-3-specific inhibitor Z-DVED-FMK (CI-3) did not prevent the initial cleavage of procaspase-3 into the p20 fragment. Therefore, corresponding with the blockade of the subsequent autocatalytic processing generating the active p17 enzyme, the level of the p20 protein increased under the treatment with Z-DVED-FMK (Figure 7A).

Another characteristic sign of apoptosis is the degradation of genomic DNA into multiple fragments referred to as DNA laddering. Electrophoretical separation of DNA isolated from DSL6A cells treated for 24 h and 48 h with CHP exhibited the typical DNA ladder, whereas DNA degradation was not detected in untreated controls (Figure 4C). Interestingly, there was no effect of CHP on the anti-apoptotic factor bcl-2 (data not shown).

Loss of adherence and survival

To verify the hypothesis that CHP-induced cell death was not simply the consequence of the loss of adherence, cell survival was quantified in suspension cultures compared with adherently growing DSL6A cells. Indeed, the rate of metabolically active cells (17%) in suspension was lower than that in the adherent counterparts (Figure 8A). However, the portion of dead cells in monolayer cultures incubated with CHP for 24 h increased to about 60% as compared to the untreated controls. Moreover, there were no significant differences between adherent and suspension cultures in CHP-treated cells (Figure 8A). Interestingly, the FAK molecule in DSL6A cells bared from adhering remained intact (Figure 8B). Taken together, our data suggest that the CHP-mediated damage of DSL6A cell functions, including detachment, growth inhibition and apoptosis, is a consequence of molecular mechanisms initiated by intracellular proteolytic processes.

DISCUSSION

In this study, we have shown that cis-hydroxyproline initiates a stress machinery in the rat pancreatic cancer cell line DSL6A, leading sequentially to growth inhibition, loss of adherence and apoptosis. The capacity of CHP to inhibit cell proliferation is consistent with the results for fibroblasts, mammary carcinoma and retinal epithelium cells[6-8].

The CHP-induced damage of DSL6A cells was associated with proteolytic fragmentation and inactivation of FAK. Various studies have suggested that FAK is essentially implicated in integrin-mediated transfer of extracellular stimuli from the cell surface across the plasma membrane, resulting in the activation of several intracellular signalling pathways[22-24]. FAK activation by phosphorylation has been shown in a variety of cell types to be dependent on integrins binding to their extracellular ligands[27]. In addition, there are several reports regarding the regulation of FAK activity by limited cleavage of the

enzyme protein. Using human platelets, Cooray *et al*[28] could identify calpain as the FAK-hydrolysing protease. In a model of oncogenic cell transformation by retroviral gene transfer of v-src, Carragher *et al*[29] showed that cell detachment is associated with calpain-dependent FAK degradation. Furthermore, focal loss of adhesion followed by apoptosis after switching off v-src is accompanied by caspase-mediated FAK proteolysis[29]. The data and the different effects of specific protease inhibitors let the authors conclude that proteolysis of FAK might be a general phenomenon associated with disassembly of focal adhesions, but can be mediated by distinct proteases under different biological conditions[29]. We have shown that in addition to FAK cleavage, the CHP-induced cell detachment, cyclin D1 decline, and caspase-3 activation in DSL6A cells could be attenuated by proteolysis inhibition. The circumvention of the different molecular and functional effects by inhibition of intracellular proteolysis suggests that protease(s) might play a superior role in the execution of CHP-mediated cell toxicity. Furthermore, CHP induced the expression of GRP78 and GADD153, both of which have been defined to indicate the UPR, a pathway of the ER stress machinery[15].

As early as 1975, Uitto *et al*[22] found that non-helical CHP-containing pro-collagen is retained in the ER of chick embryo tendon cells. However, the molecular consequences of these observations could not fully be realised at this time. The CHP-initiated impairment of DSL6A cell functions might be likely attributed to more comprehensive cellular mechanisms rather than exclusively to the disturbance of collagen synthesis, especially because DSL6A cells do not express collagen type I but only traces of collagen type IV-specific transcripts (data not shown). Thus, an interference of CHP with the synthesis of proline-rich proteins involved in striking regulatory cell functions can be assumed[31].

L-proline is able to abolish CHP-induced disturbances of DSL6A cell functions. The mutual competitive replacement of the isomeric compounds proline and CHP has to be expected[5,8]. More interestingly, the addition of L-proline could arrest and even reverse the ongoing process of the CHP-induced ER stress. The restoration is accompanied by a decrease of the expression of the stress proteins, indicating the reversibility of the UPR. GRP78 transcript levels could be achieved during about 6 h of the starting status, while the normalization of the protein concentration takes more than 2 d. According to reports by Liu *et al*[32], the delayed protein decline is probably attributed to its half life time of more than 36 h.

Intriguingly, the L-proline-induced reversion of the CHP-mediated impairment of cell functions is accompanied by a time-dependent reconstitution of the FAK molecule. Furthermore, the survival of DSL6A cells maintained in suspension cultures suggests that the proteolytic FAK degradation is not resulted from apoptosis, but occurs downstream or even constitutes a component of the ER stress cascade. In addition to the proteolytic fragmentation of FAK shown by several studies[30,29], there are reports describing ER stress-mediated activation of caspases, leading to apoptotic cell death[33]. In contrast to these data, we showed that CHP-caused DSL6A cell injury could not be influenced by inhibition of caspases, suggesting that other and/or additional protease(s) trigger CHP-induced proteolytic activity. In this context, reports should be noted which suggest the existence of a physiological role of caspases, apart from that of death executioners[33].

Based on our data, we hypothesize that CHP initiates a signal cascade including sequentially the induction of ER stress, activation of protease(s) that catalyse the caspase-independent FAK fragmentation, followed by cell detachment and finally leading to apoptosis. Further investigations are necessary to identify proteases involved in the CHP-mediated mechanisms.

Interestingly, CHP-caused FAK inactivation leading to detachment of DSL6A cells is not directly associated with apoptosis, in general the cellular response to the loss of anchorage referred to as anoikis[34]. This anoikis-resistance of the DSL6A cells can be attributed most likely to their malignant phenotype[35].

Taken together, we have shown, probably for the first time, an ER stress-mediated cytotoxic effect exerted by CHP in pancreatic cancer cells. Since the molecular equipment may vary between different cell populations, further studies are needed to characterize the susceptibility of other cell types to CHP. Lastly, in order to test the practicability of CHP in cancer therapy, studies on animal models are under way.

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BASIC RESEARCH

Expression and location of Smad2, 4 mRNAs during and after liver fibrogenesis of rats

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Abstract

AIM: To investigate the location alteration of Smad2 and Smad4 mRNAs in the liver during and after fibrogenesis in rats.

METHODS: Eighty male Wistar rats weighing approximately 200 g each were used. The rat models of experimental hepatic fibrosis were established by injection with carbon tetrachloride (CCl_4), normal rats and rats were injected with olive oil and served as control groups. *In situ* hybridization(ISH) was used to detect the Smad2 and Smad4 mRNA in liver.

RESULTS: *In situ* hybridization showed Smad2 and Smad4 mRNA expressions in the cytoplasm of hepatic stellate cells (HSC), fibroblasts and myofibroblasts around the central vein and hepatic sinus during and after fibrogenesis. Expression of Smad2, 4 mRNA was higher than that in normal and control rats.

CONCLUSION: In the process of and after hepatic fibrosis formation, HSC, fibroblasts and myofibroblasts are the major cells that express Smad2 and Smad4. The more serious the hepatic fibrosis is in the injured liver, the higher the level of Smad2 and Smad4 gene expression is during and after fibrogenesis respectively.

Key words: Smad2; Smad4; mRNA; Liver fibrogenesis

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INTRODUCTION

Fibrosis is associated with many liver diseases, including hepatitis C virus infection, iron deposition, alcohol consumption, and nonalcoholic fatty liver disease. Hepatic fibrosis results from a net increased synthesis and decreased degradation of extracellular matrix (ECM) proteins. Type Ⅰ collagen is the most prevalent ECM protein deposited[1], with activated hepatic stellate cells(HSCs) serving as the primary source. Following a fibrogenic stimulus, HSCs are activated from their normal quiescent state, whereby they increase synthesis of procollagen type Ⅰ protein[1,2], and increase cellular proliferation, migration, and contractility[3,4]. Excess ECM accumulation results in scarring within the tissue[5].

In the progression of HSCs activation, many kinds of cytokines play important roles, including transforming growth factor-beta (TGF-β)[6]. TGF-β is a multifunctional cytokine with diverse effects on development, growth, and homeostasis in most tissues[7]. Signaling by TGF-β occurs through type Ⅰ and type Ⅱ transmembrane serine/threonine kinase receptors and intracellular Smad transduction molecules. The TGF-β ligand binds to its type II receptor, either directly or via coreceptors. Once activated by TGF-β, type II receptors recruit, bind, and transphosphorylate type I receptors, thereby stimulating protein kinase activity of the later. The activated type I receptors phosphorylate Smad2 or Smad3, which then bind to Smad4. The resulting Smad complex then moves into the nucleus, where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of target genes[8].

It has been reported that there is a correlation between TGF-β and the formation of liver fibrosis[9,10]. TGF-β has a very important role in ECM production by HSC and many investigations have been done[11,12]. Antagonism of cytokines as a route to antifibrotic therapy, by blocking the interaction between TGF-β and its receptors, and the signal transduction mechanism for TGF-β, has been reported for the treatment of fibrosis[13,14]. Thus the investigation of the Smads signal transduction is indispensable for the study

of fibrosis and the development of its therapy. Until now, there have been no reports on the location and expression alteration of Smad2,4 during and after fibrogenesis. In this article, the expression and location of Smad2,4 mRNA were measured in rat livers with cRNA probes of Smad2,4 by *in situ* hybridization.

MATERIALS AND METHODS

Animal experiments

Eighty male Wistar rats weighing approximately 200 g each were used. Chronic liver injury was produced in 10 rats in each group by subcutaneous injections of 50% CCl_4 in olive oil at a volume of 2 ml/kg body weight twice weekly for 3, 6, 9, 12, 15, and 18 wk. Ten control rats similarly received olive oil alone as control group, and 10 other rats were not treated as normal group. All rats were maintained on a standard diet with water *ad libitum*. On the sixth day after the last treatment, 10 rats in each group were decapitated. Their livers were removed immediately, fixed in 4% paraformaldehyde at room temperature and snap-frozen in liquid nitrogen. The tissue specimens were stored at -80 ℃ until analysis.

Cloning of Smad2 and Smad4 cDNAs

Smad2 and Smad4 cDNAs were cloned in order to synthesize ISH riboprobes. The two cDNAs were obtained from rat liver total RNA by reverse transcription coupled to PCR by the use of the primers Smad2-up (5' GGTGGATCCGTGTCTCATCGGAAAGGG-3'), Smad2-GTGAATTCTGGAATGGAGTGGGTATAG-3') and Smad4-up (5'-AACCGGATCCATCTTCAGC ACCACCC-3'), Smad4-down (5'CGAATTCTTTGCCTA TGTGCAACCTTGC-3'), respectively. The two resulting fragments were digested with *BamH* Ⅰ and *Eco*R Ⅰ and cloned into the pSPT18 vector (Boehringer Mannheim) to obtain the pSPT18+/Smad2 and pSPT18+/Smad4 plasmids.

ISH riboprobe synthesis

The cDNA-containing plasmids were linearized as follows: pSPT18+/Smad2 and pSPT18+/Smad4 with *BamH* Ⅰ and *Eco*R Ⅰ. The linearized plasmids were then gel-isolated (Hua Shun Corp., China) and used as templates for anti-sense and sense digoxigenin (DIG)-labeled riboprobe synthesis(Boehringer Mannheim). The transcription mixture (20 μL) included 1 μg of linearized template cDNA , ATP, GTP and CTP at 1 mmol/L each, UTP 0.7 mmol/L, DIG-UTP 0.3 mmol/L, DTT 10 mmol/L, RNase inhibitor (1 U/μL of transcription mix), and T3 or T7 RNA polymerase (1 U/μL of transcription mix). Transcription was performed for at least 2 hr at 37 ℃. The template cDNAs were then digested by RNase-free DNase (2 μL at 1 U/μL, 15 min at 37 ℃), and all reactions were stopped by adjusting the reaction volume to 100 μL with EDTA (pH8.0). The riboprobes were then purified through two precipitation steps by addition of 100 μL LiCl (4 mmol/L) and 500 μL EtOH (100%), and centrifugation for 30 min at 4 ℃ in a microfuge. The pellet was resuspended in 100 μL DECP-treated water.

In situ hybridization

The serial paraffin sections (thickness 4 μm) were dried at 55 ℃, then deparaffinized by xylene and rehydrated in graded ethanol, incubated for 2×5 min in TBS containing 0.1% active DEPC (Fluka), acidified in 0.2 N HCl for 20 min at room temperature, and incubated for 2×5 min in TBS before digestion with proteinase K for 15 min, and then incubated for 2×5 min in TBS. After postfixation in 4% paraformaldehyde-PBS, sections were incubated for 2 ×5 min in TBS. And then sections were dehydrated with graded ethanol. After prehybridization at 42 ℃ for 2 h, the labeled RNA probes were added to the hybridization mix (Smad2 3.7 ng/μL, Smad4 2.3 ng/μL). The hybridization was carried out at 42 ℃ for 18 h with 20μL of hybridization mix on each section. The sections were washed with 2×SSC, 1×SSC, and TBST(TBS containing Tween20) respectively. After washed, the sections were incubated for 15 min at room temperature with Buffer I containing 1% blocking reagent (Boehringer Mannheim), and then at 37 ℃ with alkaline phosphatase-coupled anti-digoxigenin antibody(Boehringer Mannheim) diluted at 1:50 in Buffer I. Excess antibody was removed by 5×4 min washes in TBST, and the sections were equilibrated for 2× 5 min in Buffer I(Tris-HCl 100mmol/L, NaCl 100mmol/L, and $MgCl_2$ 100mmol/L, pH9.5). Color development was performed at 37 ℃ for 8 h in Buffer I containing NBT and BCIP(Promega). Staining was stopped by a 10 min washing in tap water, and non-special staining was removed in EtOH 95% with gentle agitation. Then the sections were rehydrated through successive baths of EtOH (70, 95, and 100%) and xylol (2×15 min each) and mounted in gum for microscopic examination and photography. Blank control: Smad2 and Smad4 cRNA probes for positive hepatic tissues were replaced by prehybridization solution. Negative control: *in situ* hybridization was performed in normal liver tissues. False positive control: *in situ* hybridization was performed in liver tissues injected with olive oil.

Pathology observation

The whole liver sections were stained with hematoxylin and eosin, and examined under microscope.

Statistic analysis

The *t* test was used to determine statistical significance between groups. *P* value of less than 0.05 was considered statistically significant.

RESULTS

Pathologic findings

During the hepatic fibrosis formation in the experimental rats, in liver tissues at 3 wk, infiltration of inflammatory cells was found around the portal area and central vein; at 6 wk, fatty degeneration and infiltration of inflammatory cells was detected; at 9 wk, hyperplasia of the lattice fibers and collagenous fibers was observed in portal area; at 12 wk, hyperplasia of the lattice fibers and collagenous fibers was observed in portal area and extended outwards, hyperplasia surrounding the central vein observed was

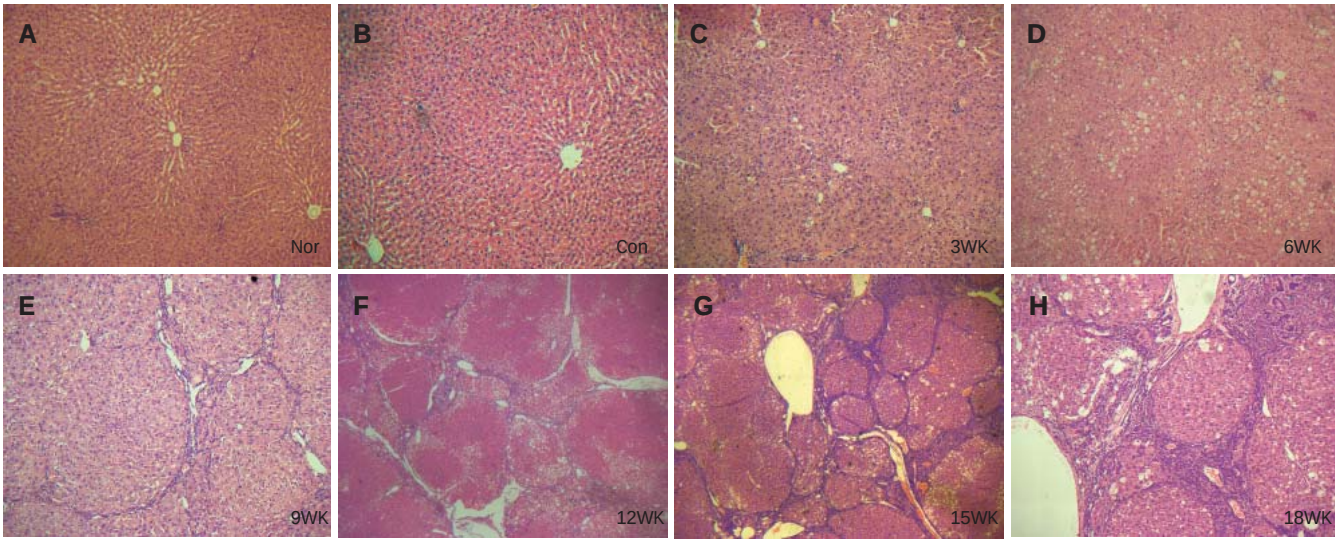

Figure 1 Pathology observation of the experimental rat liver sections stained with hematoxylin and eosin (×100). **A**: normal rats; **B**: control rats; **C**: CCl_4 treated rats at 3 wk; **D**: CCl_4 treated rats at 6 wk; **E**: CCl_4 treated rats at 9 wk; **F**: CCl_4 treated rats at 12 wk; **G**: CCl_4 treated rats at 15 wk; **H**: CCl_4 treated rats at 18 wk.

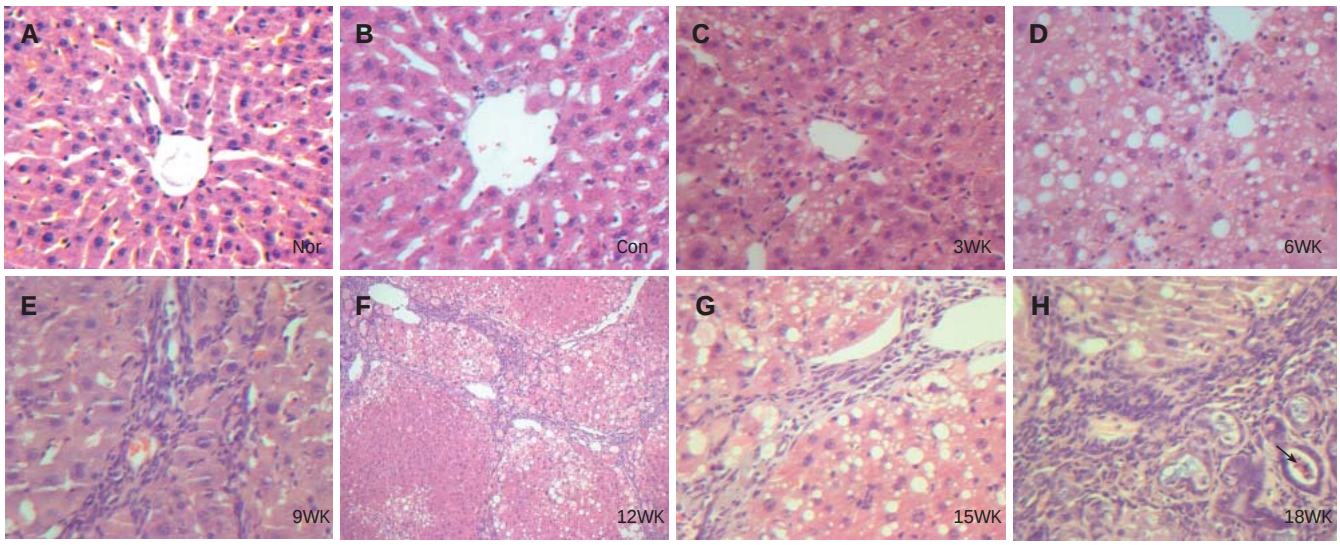

Figure 2 Pathology observation of the experimental rat liver sections stained with hematoxylin and eosin (×400). The arrow shows atypical hyperplasia in the epithelium of bile capillary. **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, see Figure 1.

distributed along hepatic sinus and connected with each other, the hepatic lobules were encysted and separated by collagen bundles, the normal structure of lobules was destroyed, and pseudolobules formed, infiltration of inflammatory cells was found around the portal area and central vein; at 15 and 18 wk, excluding the findings of 12 wk, atypical hyperplasia was found in the epithelium of biliary capillary. The structure of liver tissues was normal in normal and control groups (Figures 1 and 2).

Smad2 and Smad4 location

Smad2 and Smad4 mRNAs in liver from experimental rats were detected mainly in the HSCs, fibroblasts and myofibroblasts around the portal area and central vein. Expressions of Smad2 and Smad4 mRNAs exhibited as blue particles in cytoplasm. No positive expression was found in nuclei. There was only mild positive expression in normal and control groups. Image pattern analysis showed that the expression in the experimental group was much stronger than that in the normal and control groups (Table 1, Figures 3 and 4).

DISCUSSION

To establish the model of liver fibrosis successfully is the key for detection of Smad2 and Smad4 mRNAs in the liver tissue during and after fibrogenesis. Researches for establishing the model of liver fibrosis with CCl_4 began in 1936. After that many methods to establish the model of liver fibrosis have been tried. Among them, liver fibrosis models induced by immune response or CCl_4 have been generally accepted mainly because they are more close to the disease characteristics in terms of the distinct stages of the disease of human body and a low mortality. In this study, the method of injecting CCl_4 was used to establish the model of liver fibrosis, and it was proved successful

Table 1 Expression of Smad2 and Smad4 mRNA during and after liver fibrogenesis (mean ± SD)

Group	*n*	Nor	Con	3wk	6wk	9wk	12wk	15wk	18w
Smad2	80	120.20±9.00	113.30±15.18	150.50±8.13^{ce}	213.40±13.92^{ace}	310.30±25.43^{ace}	510.80±7.53^{ace}	523.50±17.35^{ace}	568.40±7.64^{ace}
Smad4	80	99.40±3.65	105.40±10.89	143.50±6.47^{ce}	165.00±32.10^{ace}	305.10±18.53^{ace}	485.00±4.08^{ace}	500.40±10.94^{ace}	529.00±6.78^{ace}

$^{a}P<0.05$ *vs* a previous time point, $^{c}P<0.05$ *vs* normal group, $^{e}P<0.05$ *vs* control group.

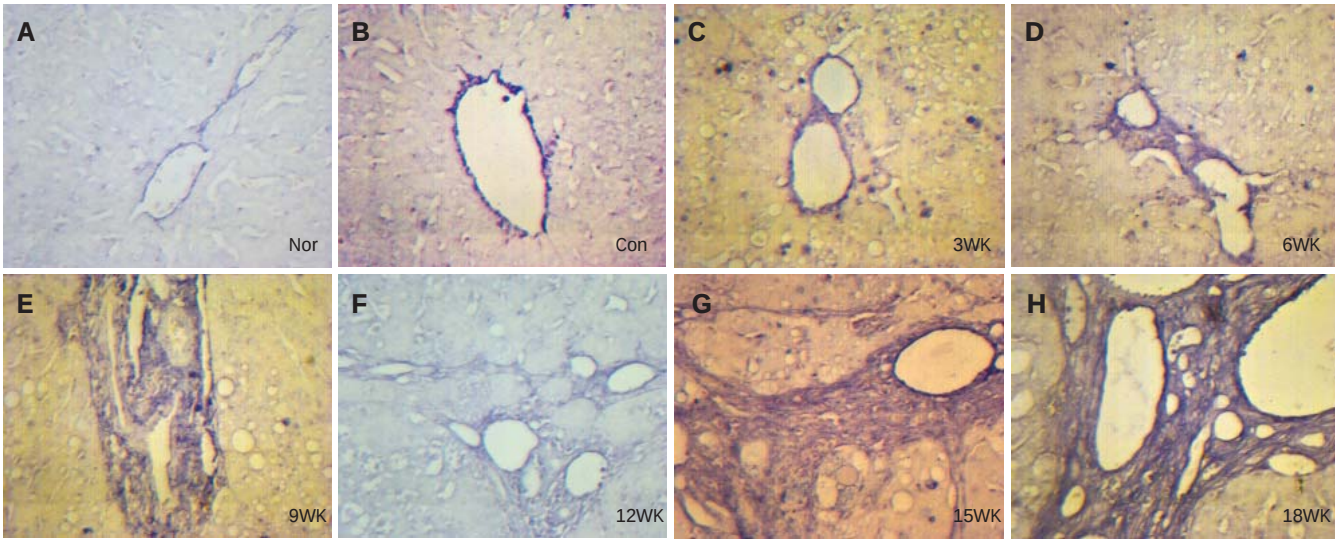

Figure 3 Expression of Smad2 mRNA in experimental rats liver sections during and after fibrosis (*in situ* hybridization ×400). **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, see Figure 1.

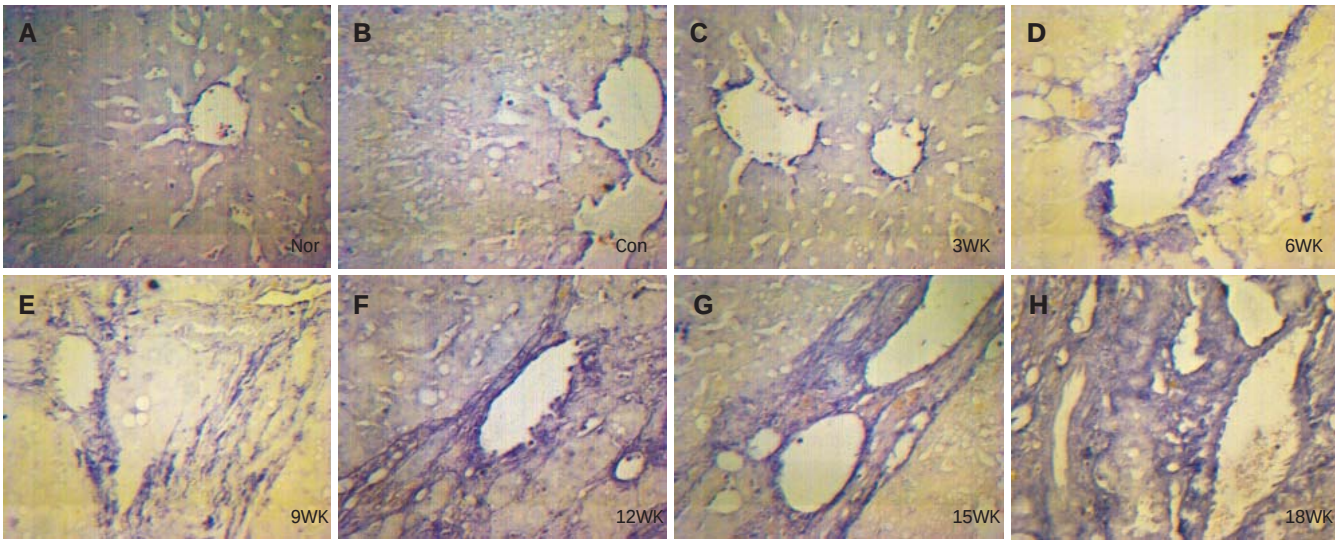

Figure 4 Expression of Smad4 mRNA in experimental rats liver sections during and after fibrosis (*in situ* hybridization ×400). **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, see Figure 1.

by histochemical analysis of the sections stained with hematoxylin and eosin. According to the pathologic findings of this study, the exact time for establishment of rat hepatic cirrhosis by injecting CCl_4 may be at 12 wk. From 15 and 18 wk on, atypical hyperplasia was found in the epithelium of bile capillary. It may indicate that injecting CCl_4 may cause hepatic carcinoma.

TGF-β consists of a big molecular family that has a common structure including BMP and activin. These proteins have many roles in the cell structure and function, such as cell growth, differentiation, apoptosis and regulation of ECM production[15,16]. The main functions of the TGF-β super family are morphogenesis, inflammation, tissue recovery and oncogenesis[17]. Smads are intracellular signal transductive molecules of the TGF-β super family. These molecules were discovered as Mothers against dpp (Mad)[18,19], or sma[20] and named Smad in 1996[21]. According to the differences of structure and function, nine Smads have been reported and classified into three groups[7,22]. Smads 2 and 3 are named R-Smads in the

pathway and Smad 4 a Co-Smads for all these pathways. Smads 6, 7, 8 and Xsmad 8 are inhibitory factors of these Smads. When TGF-β binds to its receptor, Smad 2/3 is phosphorylated and binds with Smad 4, together they move into the nucleus for translation and expression of the target gene[7,23]. These mechanisms are thought to play a role in the process of liver damage, recovery, as well as liver fibrosis. In a damaged liver including chronic inflammation, in hepatitis B, TGF-β signaling was reported[24]. Not only Kupffer cells but also the HSC produce TGF-β by the autocrine system[25-28]. TGF-β1 stimulation evokes ECM production, which develops into liver fibrosis.

In this study, Smad2 and Smad4 mRNAs were investigated in rat liver during and after fibrogenesis caused by CCl_4 *in vivo*. With the method of *in situ* hybridization, we found Smad2 and Smad4 mRNAs were expressed obviously in HSC, myofibroblasts and fibroblasts of livers of liver fibrosis group, mostly in portal area and fibrous septum, while very mildly in vascular endothelial cells of livers from normal and control groups. While with the development of liver fibrosis, expressions of Smad2 and Smad4 mRNAs were increased also. These results indicate that Smad2 and Smad4 play an important role in the progression of liver fibrosis. In the sections of liver fibrosis at 15 and 18 wk, expressions of Smad2 and Smad4 mRNAs were higher in the epithelium of bile capillary than in normal and control groups. At this stage, there was atypical hyperplasia in the epithelium of bile capillary, indicating early stage of carcinoma. Therefore, Smad2 and Smad4 may be associated with cancerogenesis.

After stimulation of TGF-β, Smad2, 3 and 4 proteins translocate from cytoplasm to nucleus in MV1Lu cells, analyzed by immunofluorescence using specific antiserum[22]. Moreover, Smad3 seems to play the most important role in liver fibrosis, because Smad3 interacts with SP1 which increases expression of ECM by regulating COL1A2 gene[29]. By transfection of Smad3 expression plasmid in CF37 cells, ECM also increases by regulating COL1A2 gene. Nevertheless, COL1A2 transcription in CF37 cells is markedly increased by TGF-β after transfection with a Smad2 expression plasmid[30]. So Smad2 also plays an important role in liver fibrosis. Our findings are consistent with the above results. The difference is perhaps due to use of different cell lines or experimental conditions. Further researches are needed to confirm the results.

As the liver fibrosis is a complex process, involving many cytokines, cells, and signal transduction pathways, the mechanism cannot be fully explained by these experiments. However, it is clear that TGF/Smads signal transduction pathway plays an important role in liver fibrosis which has been proved by many researches. In our study, Smad2 and Smad4 mRNAs were detected in the plasma of HSC, fibroblasts and myofibroblasts around the portal area and central vein by *in situ* hybridization. Expressions of Smad2 and Smad4 mRNAs were higher during and after liver fibrogenesis than normal and control groups, indicating that they play an important role in liver fibrosis.

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CLINICAL RESEARCH

Expression of *c-kit* receptor in human cholangiocarcinoma and *in vivo* treatment with imatinib mesilate in chimeric mice

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Abstract

AIM: To investigate the *c-kit* expression in biliary tract cancer cell lines and histological sections from patients with extrahepatic cholangiocarcinoma (CC) and to evaluate the efficacy of *in vitro* and *in vitro* treatment with imatinib mesilate.

METHODS: The protein expression of *c-kit* in the human biliary tract cancer cell lines Mz-ChA-2 and EGI-1 and histological sections from 19 patients with extrahepatic CC was assessed by immunoblotting, immunocytochemistry, and immunohistochemistry. The anti-proliferative effect of imatinib mesilate on biliary tract cancer cell lines Mz-ChA-2 and EGI-1 was studied *in vitro* by automated cell counting. In addition, immunodeficient NMRI mice (Taconic™) were subcutaneously injected with 5 x 10^6 cells of cell lines MzChA-2 and EGI-1. After having reached a tumour volume of 200 mm^3, daily treatment was started intraperitoneally with imatinib mesilate at a dose of 50 mg/kg or normal saline (NS). Tumor volume was calculated with a Vernier caliper. After 14 d, mice were sacrificed with tumors excised and tumor mass determined.

RESULTS: Immunoblotting revealed presence of *c-kit* in Mz-ChA-2 and absence in EGI-1 cells. Immunocytochemistry with *c-kit* antibodies displayed a cytoplasmatic and membraneous localization of receptor protein in Mz-ChA-2 cells and absence of *c-kit* in EGI-1 cells. *c-kit* was expressed in 7 of 19 (37%) extrahepatic human CC tissue samples, 2 showed a moderate and 5 a rather weak immunostaining. Imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in the *c-kit* positive cell line Mz-ChA-2 (31%), but not in the *c-kit* negative cell line EGI-1 (0%) ($P < 0.05$). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both cell lines (51% *vs* 57%). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect on both cell lines. The IC_{50} values were 9.7 μmol/L and 11 μmol/L, respectively. After 14 d of *in vitro* treatment with imatinib mesilate, using the chimeric mouse model, *c-kit* positive Mz-ChA-2 tumors had a significantly reduced volume and mass as compared to NS treatment ($P < 0.05$). In contrast to that, treatment of mice bearing *c-kit* negative EGI-1 tumors did not result in any change of tumor volume and mass as compared to NS treatment.

CONCLUSION: *c-kit* expression is detectable at a moderate to low protein level in biliary tract cancer. Imatinib mesilate exerts marked effects on tumor growth *in vitro* and *in vitro* dependent on the level of *c-kit* expression.

Key words: Cholangiocarcinoma; Imatinib; Tyrosine kinase inhibitor; c-kit; Chimeric mice

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INTRODUCTION

Protein kinases are functionally involved in a broad variety of human cancer[1,2]. In tumor cells, it is common that key tyrosine kinases are no longer adequately controlled, and excessive phosphorylation sustains signal transduction pathways in an activated state. Receptor tyrosine kinases (RTKs) are membrane bound proteins, consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment, and a cytoplasmic part harboring the protein tyrosine kinase activity. Ligand-induced dimerization, resulting in autophosphorylation of their cytoplasmic domains, is the major mode of activation of RTKs[3]. Many oncogenic mutations in RTKs involve either point mutations leading to constitutive dimerization of the receptors or translocations causing fusion of oligomeriza-

tion motifs to RTKs. Overexpression, probably caused by mutations in gene regulatory sequences or gene amplification, may lead to constitutive dimerization of RTKs. Moreover, mislocalization of RTKs due to fusion to other proteins may contribute to their oncogenic phenotype. Phosphorylated tyrosine residues in the receptor tails then function as recruitment sites for downstream signalling proteins containing phosphotyrosine-recognition domains, such as the SRC homology 2 (SH2) domain or the phosphotyrosine-binding (PTB) domain. These molecules act as relay points for a complex network of independent signalling molecules that ultimately affect gene transcription within the nucleus. *c-kit* (CD-117) is a transmembrane tyrosine kinase receptor in which the extracellular protein binds a ligand known as stem-cell factor (also known as Steel factor) and the intracellular portion contains the actual kinase enzymatic domain[4-6]. *c-kit* is similar in structure to several other RTKs with oncogenic capabilities, including platelet-derived growth factor receptor (PDGF-R) A and B, colony stimulating factor 1 receptor (CSF1-R), and fms-related tyrosine kinase 3 receptor (FLT3-R). *c-kit* is expressed at high levels in hematopoietic stem cells, mast cells, melanocytic cells, germ cells, and the interstitial cells of Cajal (ICC)[7-10]. The receptor forms homodimers upon ligand binding leading to receptor activation. This triggers activation of critical downstream signalling pathways, such as Ras/Raf/mitogen-activated protein kinase kinase (MAPKK)/mitogen-activated protein kinase (MAPK) (cell proliferation) and phosphatidylinositol 3-kinase (PI3K)/AKT (cell survival)[11,12]. There are receptor activating oncogenic *KIT* mutations which involve the extracellular (exon 9), juxtamembrane (exon 11) and kinase domains (exons 13 and 17). As a consequence, in hematologic neoplasms, the exact signalling pathways activated by the mutant *KIT* differ from those activated by normal *KIT*[13,14].

The clinical development of targeted tyrosine kinase inhibitors represents a breakthrough for cancer treatment. They are designed to take advantage of the molecular differences specific to tumor cells compared with normal tissues. The goal is to achieve tumor responses with better safety profiles than those associated with cytotoxic chemotherapies that exhibit narrow therapeutic indices and little selectivity for cancer cells over normal proliferating cells. Imatinib mesilate (STI-571, Gleevec™, Novartis), a 2-phenylaminopyrimidine, was primarily designed to treat chronic myeloid leukemia (CML) by inhibiting bcr-abl fusion protein, created by the t(9; 22) chromosomal translocation, which generates the distinctive Philadelphia (Ph) chromosome[15]. However, imatinib mesilate is not a specific bcr-abl inhibitor, its action extends to c-abl receptor, PDGF-R, and *c-kit*-R[15]. Moreover, it promotes NK cell activation[16] and downregulates telomerase activity[17]. Therefore, imatinib mesilate has been evaluated for the therapy of gastrointestinal stromal tumors (GISTs), because most of them characteristically show *c-kit* overexpression. For metastatic GISTs, imatinib mesilate is nowadays the therapeutical standard, based on a phase II study, including 147 patients with unresectable GISTs, which reported a partial response rate of 54 % and a rate of stable disease of 28 %[18]. A closer inspection of the data clearly displayed that *KIT* mutational status correlated with response to imatinib mesilate. Thus patients with activating *KIT* mutations in exon 11 had a partial response of close to 80%. In contrast, patients whose tumors expressed wild-type *KIT*, with no mutations, had a response rate of only 18%[19]. However, there are only a few published phase I and II studies investigating the effect of imatinib mesilate on gastrointestinal tumors, others than GISTs. Whereas three studies detected a modest level of biological effect of imatinib in hepatocellular carcinoma (HCC) and carcinoid tumor, there was no effect for the treatment of pancreatic carcinoma in two other studies[20-24]. In this study we investigated *c-kit* protein expression in biliary tract cancer cell lines and histological sections from 19 patients with extrahepatic cholangiocarcinoma (CC) and evaluated the efficacy of *in vitro* and *in vivo* treatment with imatinib mesilate.

MATERIALS AND METHODS

Materials

Human gallbladder cancer cell line Mz-ChA-2[25], human extrahepatic cholangiocarcinoma cell line EGI-1[26], and colorectal carcinoma cell line HT-29[27] were cultured as mono-layers in Dulbecco`s modified Eagle`s medium (DMEM, Invitrogen GmbH Karlsruhe, Germany) supplemented with 100 mL/L fetal bovine serum (FBS, Invitrogen GmbH Karlsruhe, Germany), 100 ku penicillin and 100 g/L streptomycin in humidified atmosphere of 900 mL/L air and 100 mL/L CO_2 at 37 ℃. Small cell lung cancer (SCLC) cell line NCI-H69[28] was kept in RPMI 1640 medium (Invitrogen GmbH Karlsruhe, Germany) containing 100 mL/L FBS and antibiotics in humidified atmosphere of 950mL/L air and 50mL/L CO_2 at 37 ℃. The 2-phenylaminopyrimidine derivative STI-571 (imatinib mesilate, Gleevec™) was provided by Novartis Pharma AG (Basel, Switzerland). Stock solution (10 g/L) was prepared in normal saline (NS) and stored at -20 ℃. Hoechst dye was purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), rabbit polyclonal c-kit antibody (CD117 Ab-6) from Neomarkers (Dunn Labortechnik GmbH, Asbach, Germany), and Cy™3-conjugated donkey anti-rabbit antibody from Jackson ImmunoResearch (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, United Kingdom). Six to eight-week-old female athymic NMRI nude mice were supplied from Taconic (Taconic Europe, Ry, Denmark) and held under pathogen-free conditions. Human care was administered, and study protocols complied with the institutional guidelines.

Immunoblotting

Cell culture mono-layers were washed twice with ice-cold PBS and lysed with buffer containing Tris-HCl (20 mmol/L, pH 7.5), NP-40 (10 g/L), Triton-X (5 g/L), NaCl (250 mmol/L), EDTA (1 mmol/L), 100 ml/L glycerol, and one tablet of complete mini-EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany) (in 10 mL buffer). Protein concentration was determined by the Bradford protein assay (Bio-Rad, Munich, Germany). 30 μg of cell lysates were separated on SDS-polyacrylamide gels and electroblotted onto poly-

vinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution (50 g/L BSA in 10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20) (TBS-T), followed by incubation with the primary antibody at 4 ℃ overnight. The membranes were then washed in TBS-T and incubated with HRPO-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction.

Immunocytochemistry

For immunocytochemistry 1 x 10^5 tumor cells were seeded on coverslips of 12-well plates and incubated for one day. Then, medium was removed, cells were washed with PBS and fixed with 8 g/L formaldehyde in PBS for 2 h, washed again, and then permeabilized in 3 g/L Triton-X and 10 g/L DMSO in PBS for another 10 min. After nonspecific antibody binding was blocked by incubation with 50 ml/L donkey serum in 3 g/L Triton-X and 10 g/L DMSO in PBS at room temperature for 2 h, the primary antibody was added, followed by an incubation at 4 ℃ overnight. The omission of primary antibody served as negative control. The next day, cells were washed again and probed with Cy^{Tm}3-conjugated donkey anti-rabbit antibody at room temperature for 2 h. Then, extensive washing and nuclear staining was performed using Hoechst dye according to manufacturers instructions. Finally, coverslips were mounted on slides with 900 ml/L glycerol and slides were analyzed with a Zeiss LSM 510 laser scanning microscope (Carl Zeiss Jena GmbH, Jena, Germany).

Immunohistochemical staining

Paraffin-embedded tissue sections from resection specimens of 19 patients with extrahepatic CC were de-waxed, hydrated through graded alcohol, and immunostained with antibodies to c-kit using the avidin-biotin horseradish peroxidase method as previously described[29]. Immunoreactivity was revealed using True Blue^{TM} peroxidase substrate (KPL, Gaithersburg, USA). The sections were counterstained with hematoxilin and mounted with Permount (Fisher, Pittsburg, USA). For the tumor tissues, the percentage of positive cells was estimated and the staining intensity was semiquantitatively recorded as 1+, 2+, or 3+. For statistical analyses, the staining results were categorized into four groups according to Went *et al*[30]. Tumors without any staining were considered negative. Tumors with 1+ staining intensity in less than 60% of cells and 2+ intensity in less than 30% of cells were considered weakly positive. Tumors with 1+ staining intensity in ⩾ 60% of cells, 2+ intensity in 30% to 79%, or 3+ intensity in less than 30% were considered moderately positive. Tumors with 2+ intensity in ⩾ 80% or 3+ intensity in ⩾ 30% of cells were considered strongly positive. Only membranous or membranous plus cytoplasmic staining was considered for analysis because cytoplasmic staining alone proved to be false-positive in all preabsorption control experiments.

Inhibition of cell growth

The effect of imatinib treatment on cellular proliferation was assessed by automated cell counting (Schaerfe Casy 2.0 Cell Counter, Reutlingen, Germany) according to the manufacturers instructions. Briefly, 2 × 10^5 cells were seeded in T-25 cell culture flasks. Twenty-four hours after incubation, cells were treated with imatinib mesilate at 5 different concentrations (0, 1, 5, 10, and 20 μmol/L, respectively). After 6 d of incubation, cells were trypsinized, washed, and analyzed in triplicates by automated cell counting.

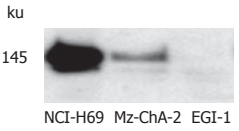

Figure 1 c-kit protein expression in biliary tract cancer cell lines.

Animal studies

Tumors were induced by injecting 5×10^6 Mz-ChA-2 or EGI-1 cells in 200 μL PBS sc into the flank region of NMRI nude mice (n = 40). Treatment was started when an average tumor volume of 200 mm³ was reached (usually after 2 wk). The verum group (n = 2×10) received imatinib mesilate, dissolved in normal saline (NS), ip once a day at 50 mg/kg, whereas the control group (n = 2×10) received NS only. Treatment was continued for 14 consecutive days, tumors were daily measured with a Vernier caliper and tumor volumes were calculated using the formula tumor volume = 0.5 × L × W^2, where L represents the length and W the width of the tumor. When treatment was finished, animals were sacrificed with tumors excised and weighed.

Statistical analysis

Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean value with standard deviation (SD). Inter-group comparisons were performed with the Student t test. P< 0.05 was considered significant.

RESULTS

c-kit protein expression in biliary tract cancer cell lines

The expression of *c-kit* in both human biliary tract cancer cell lines Mz-ChA-2 and EGI-1 was assessed by immunoblotting. Cell lysate of SCLC cell line NCI-H69, which has been reported previously to express *c-kit*[28], served as positive control and showed an intense band at about 145 kDa, corresponding to *c-kit* receptor (Figure 1). In accordance with our previous study of *c-kit* m-RNA expression[31], immunoblotting of Mz-ChA-2 cell lysate demonstrated *c-kit* protein expression in this cell line as well. In contrast, *c-kit* expression was not detected in the m-RNA negative cell line EGI-1 (Figure 1). To further analyze *c-kit* expression in these cell lines, immunocytochemistry was performed. In this experiment, colorectal cancer cell line HT-29, which is known to express *c-kit*, served as positive control[27]. Immunocytochemical analysis showed a strong membraneous staining of the receptor protein in this cell line (Figure 2). In the tested biliary tract cancer cell lines, staining of *c-kit* revealed both cytoplasmatic and membraneous localization of receptor protein in Mz-

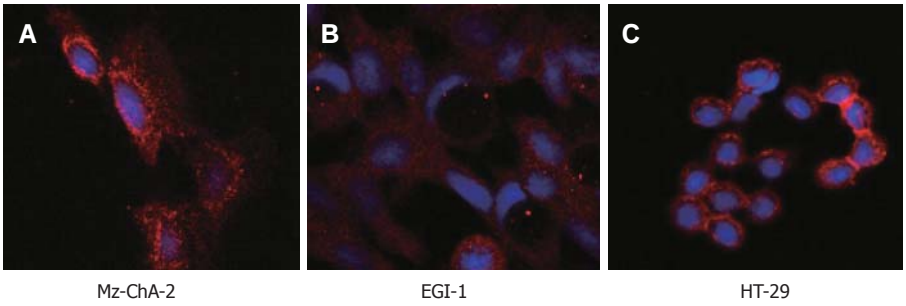

Figure 2 Immunocytochemistry in biliary tract cancer cell lines of Mz-ChA-2 (**A**), EGI-1 (**B**) and HT-29 (**C**).

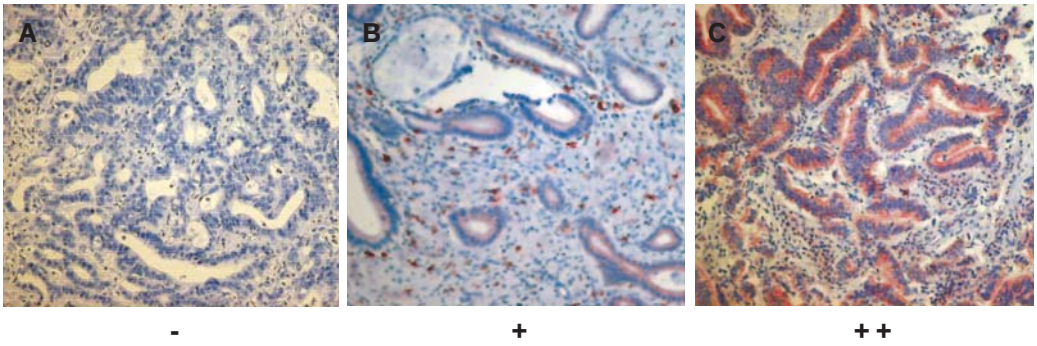

Figure 3 c-kit protein expression in 12 (**A**), 5 (**B**) and 2 (**C**) human biliary tract cancer tissue samples (SABC x 200).

ChA-2 cells and absence of *c-kit* in EGI-1 cells (Figure 2).

c-kit protein expression in human biliary tract cancer tissue

In order to analyze the expression of c-kit in human biliary tract cancer, paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with c-kit-antibodies using the avidin-biotin horseradish peroxidase method. The clinical characteristics of the study population were as follows: median age of patients was 61 years (range, 39-74 years); UICC IA, IB, IIA, IIB, III, and IV tumor stage (UICC 2002)[32] had 1, 3, 7, 6, 1, and 1 patients, respectively; Bismuth-Corlette[33] types I, II, IIIA, IIIB, and IV were found in 1, 1, 8, 2, and 7 patients, respectively; and 16 patients had well or moderately differentiated tumors. As a result, 2 of the 19 tissues samples displayed a moderate immunostaining (++), 5 a rather weak immunostaining (+), and 12 were negative (-). Thus, 7 of 19 (37%) of the histological sections of extrahepatic hilar CC tested showed moderate to weak expression of c-kit. The immunoreactivity of c-kit was found mainly in the cell membrane (Figure 3). Highly-differentiated tumors seemed to show a higher level of c-kit expression than low-differentiated tumors.

Inhibition of cellular growth by imatinib mesilate

To assess the effect of imatinib mesilate treatment on cellular proliferation of both biliary tract cancer cell lines, automated cell counting was performed, using the colorectal carcinoma cell line HT-29 as a positive control[27]. After 6 d of incubation, imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in *c-kit* positive cell line Mz-ChA-2 (31%±7%), a moderate growth inhibition in *c-kit* positive cell line HT-29 (7%±9%), and no growth inhibition in *c-kit* negative cell line EGI-1 (0%±10%) ($P<0.05$, Mz-ChA-2 *vs* EGI-1). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both biliary tract cancer cell lines significantly (51%±4% *vs* 57%±4%, Mz-ChA-2 *vs* EGI-1). However, this effect was even stronger for HT-29 (91%±19%) ($P<0.05$, HT-29 *vs* Mz-ChA-2 and EGI-1). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect in all cell lines (99%±1% *vs* 99%±1% *vs* 100%, Mz-ChA-2 *vs* EGI-1 *vs* HT-29) (Figure 4). The resulting IC_{50} values were 9.7 μmol/L, 11 μmol/L, and 9.5 μmol/L, respectively.

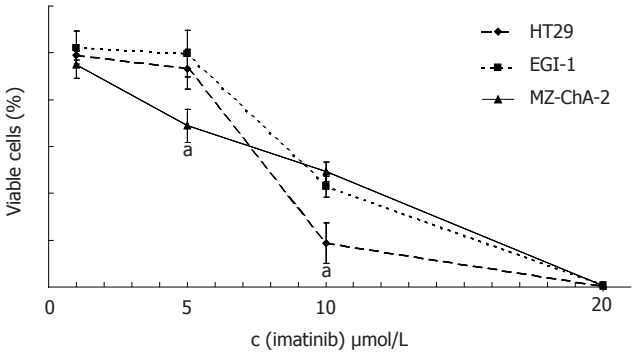

Figure 4 Inhibition of imatinib on cell growth ($^aP<0.05$).

Inhibition of tumor cell growth by imatinib mesilate in nude mice

To assess the antitumor activity of imatinib mesilate *in vivo*, tumors were induced in nude mice by subcutaneous injection of either Mz-ChA-2 or EGI-1 cells into the flanks of the animals ($n=20$ for each cell line). Treatment of mice consisted of daily ip injections of 50 mg/kg BW imatinib mesilate, and injection of NS served as control.

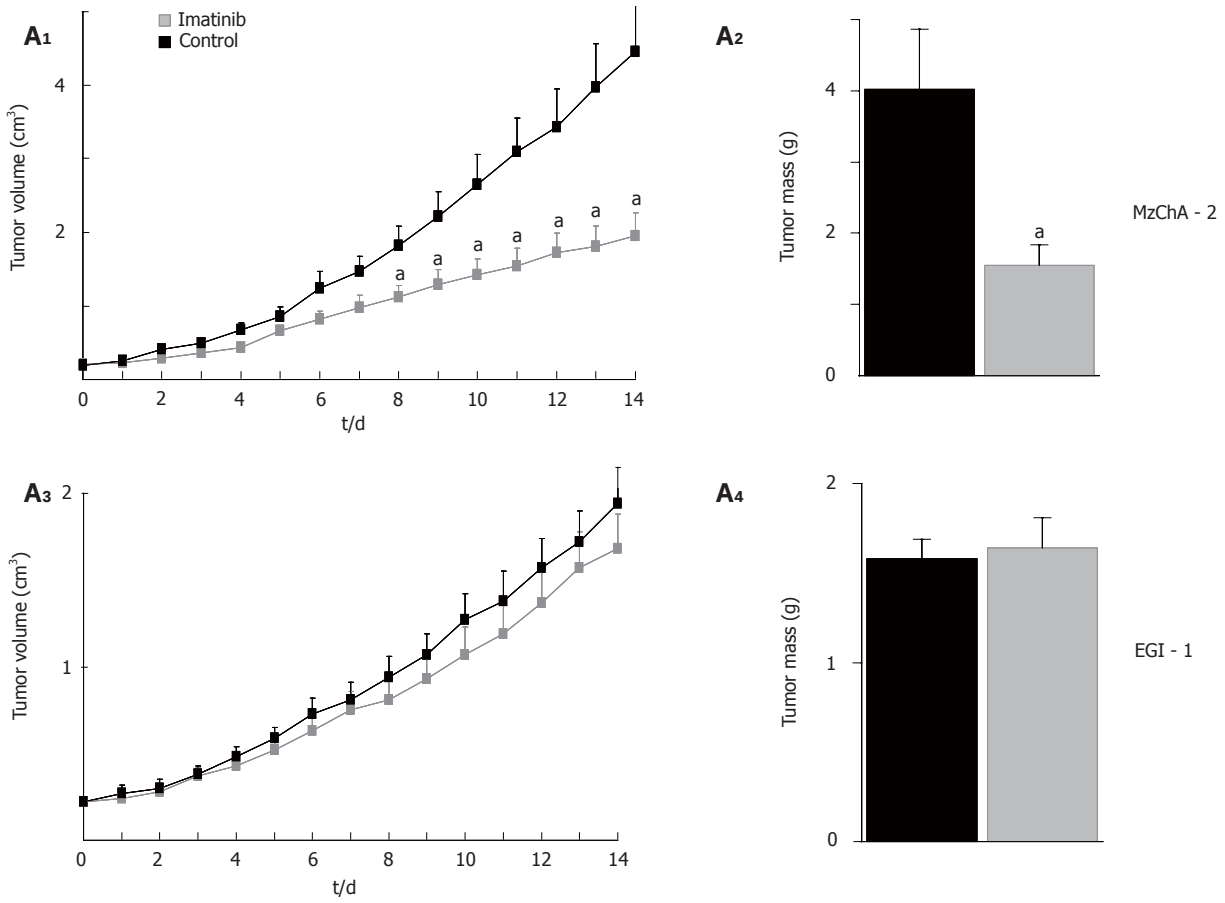

Figure 5 *In vivo* treatment with imatinib mesilate in chimeric mice; **A**: effect on tumor volume and tumor mass ($^aP<0.05$, imatinib *vs* control); **B**: hematoxilin-eosin stained paraffin-embedded tissue sections.

Eight to 14 d after beginning of treatment, Mz-ChA-2 cell tumors had a significantly reduced volume in comparison to control ($P<0.05$). At the end of the experiment (d 14) tumor mass was significantly diminished as compared to control ($P<0.05$). In contrast to that, imatinib mesilate treatment of mice bearing EGI-1 cell tumors did not result in any change of tumor growth compared to control (Figure 5A). Hematoxilin-eosin stained paraffin-embedded tissue sections from mouse tumors showed the typical features of low-differentiated adenocarcinoma (Figure 5B).

DISCUSSION

Non-resectable biliary tract cancer is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy. It is therefore essential to search for new therapeutical approaches. Imatinib mesilate (STI571, Gleevec™), which was found to inhibit the *bcr-abl* tyrosine-kinase resulting from the translocation t(9;22) in chronic myeloid leukemia (CML), as well as *c-kit* and PDGF-R tyrosine kinases, may be an alternative[34]. We have previously shown expression of *c-kit* and PDGF-R m-RNA in 50% and 75% of 12 biliary tract cancer cell lines[31]. Activating mutations of *KIT* were not found, which is consistent with the data of Sihto *et al*[35]. This group screened 334 human cancers (32 histologic tumor types) for mutations with sensitive denaturing high-performance liquid chromatography (DHPLC) and found *KIT* mutations only in GIST tumors. In our study, *c-kit* receptor ligand SCF was present in all cells lines, respectively. We reasoned that the proliferation of these cells might be stimulated by an autocrine mechanism. In order

to test this hypothesis, we treated cell lines with different concentrations of imatinib mesilate. Imatinib mesilate at an intermediate concentration of 10 μmol/L and a higher concentration of 20 μmol/L reduced survival in all cell lines examined by manual cell counting after staining with trypan blue. The reduction was statistically significant higher in *c-kit* positive cell lines ($P < 0.02$) and was independent from PDGF-R status. At 50 μmol/L, a general non-specific toxic effect occurred in all cell lines. The average IC_{50} for growth inhibition of *c-kit* positive cell lines was estimated to be 10 μmol/L. 5-Fluorouracil (5-FU) alone (0.1 μg/mL) reduced cell growth by 40 % - 50 %, while the combination with imatinib mesilate reduced cell growth by another 20% (1 μmol/L) ($P = 0.008$) and 30% (10 μmol/L) ($P = 0.0001$), respectively. Additionally, treatment of cells with imatinib mesilate ± 5-FU had no modulating effects on S-phase fraction, but was associated with a significant induction of apoptosis[31].

However, at that time it was unclear whether *c-kit* could be detected at the protein level and how its distribution whithin the tumor cells was. Moreover, while this work was in progress, Chiorean *et al* were unable to detect *c-kit* at protein levels in human cholangiocarcinoma cell lines KMCH-1 and Mz-CHA-1[36]. In the present study, immunoblotting confirmed the results of our previous m-RNA analysis for Mz-ChA-2 and EGI-1 cells. Immunocytochemistry with *c-kit* antibodies displayed a cytoplasmatic and membraneous localization of receptor protein in *c-kit* positive Mz-ChA-2 cells, indicating a steady receptor turnover. Cell lysates of SCLC cell line NCI-H69 and colorectal cancer cell line HT-29 served as positive controls for immunoblotting and immunocytochemistry. Both cell lines displayed a stronger protein expression that might correlate with a higher sensitivity for imatinib mesilate treatment as shown in previous studies[27,28,37,38]. In order to verify that *c-kit* protein is not only expressed in cholangiocarcinoma cell lines, but also in human biliary tract cancer tissue, paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with *c-kit*-antibodies using avidin-biotin horseradish peroxidase method. *c-kit* was expressed in 7 of 19 (37%) extrahepatic human CC tissue samples, 2 showed a moderate and 5 a rather weak immunostaining. This result is comparable to a previous analysis of 13 patients with intrahepatic cholangiocarcinoma which demonstrated an expression level of 31%[39]. In contrast, a study from Argentina detected only 6% *c-kit* expression in 50 specimens from gallbladder carcinoma[40]. Moreover, a weak to moderate *c-kit* expression was shown in 7 of 12 (58%) bile duct and gallbladder adenocarcinomas by Aswad *et al*[41]. Finally, a recent multitumor tissue microarray (TMA) analysis by Went *et al*, containing more than 3500 paraffin-embedded tumor samples representing more than 120 tumor types and subtypes, and a recent TMA analysis by Sihto *et al* showed negative results for gallbladder carcinoma ($n = 27$) and cholangiocarcinoma ($n = 3$)[30,35]. These controversial data could be explained by different antibodies used or different tissue selections. Using automated cell counting, we tried to confirm our manual cell counting data for the *c-kit* positive cell line Mz-ChA-2 and the *c-kit* negative cell line EGI-1 after treatment with imatinib mesilate. Imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in *c-kit* positive cell line Mz-ChA-2 (31%), but not in *c-kit* negative cell line EGI-1 (0%) ($P < 0.05$). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both cell lines (51% *vs* 57%), but was most effective in colorectal carcinoma cell line HT-29 (91%). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect in both cell lines. The results for these particular cell lines indicate that: (1) at a concentration of 5 μmol/L imatinib mesilate seems to exhibit a *c-kit* receptor dependent cell growth inhibition. The different IC_{50} values of 9.5 μmol/L for HT-29, 9.7 μmol/L for Mz-ChA-2, and 11 μmol/L for EGI-1 may therefore be influenced by the different level *of c-kit* expression; (2) at a concentration of 10 and 20 μmol/L the inhibition by imatinib mesilate seems to be no longer *c-kit* receptor dependent. A possible explanation may be a significant decrease in epidermal growth factor receptor (EGF-R) and focal adhesion kinase (FAK) phosphorylation at these concentrations, as observed by Chiorean *et al* for cholangiocarcinoma cell line KMCH-1 which was associated with a reduction in Akt activity resulting in loss of Mcl-1, a potent anti-apoptotic Bcl-2 family member[36]. Whether an additional dose dependent inhibition of telomerase activity, as observed for *c-kit* positive Ewing sarcoma, melanoma, myeloma, breast cancer, Fanconi anemia, and *c-kit* negative murine pro-B cells, plays a role in cholangiocarcinoma has not been evaluated yet[17]; (3) the assumed general toxic effect of imatinib mesilate at a concentration of 20 μmol/L indicates that even a *c-kit* and PDGF-R negative cell line like EGI-1 can sometimes be destroyed at this concentration whereas our previous meta-analysis of 6 *c-kit* m-RNA negative biliary tract cancer cell lines demonstrated a general toxic effect only at 50 μmol/L with a potential inhibition at 20 μmol/L[31].

Even more important are therefore the results from our chimeric mouse model since so far there has been no animal model published for *in vivo* treatment with imatinib mesilate in biliary tract cancer. At the end of a 14 d treatment period with imatinib mesilate, *c-kit* positive Mz-ChA-2 tumors had a significantly reduced volume and mass as compared to NS treatment ($P < 0.05$). In contrast to that, treatment of mice bearing *c-kit* negative EGI-1 tumors did not result in any change of tumor volume and mass as compared to NS treatment. These results support the notion of a *c-kit* receptor expression dependency of sensitivity to imatinib mesilate *in vivo*. At the selected dose of 50 mg/kg, which was chosen according to a study by Druker *et al*[42], no toxic side effects occurred. As discussed by Druker *et al*[43], at steady state, a once-daily administration of 400 mg imatinib mesilate, the commonly used oral dose, translates into a mean maximal serum concentration of 4.6 μmol/L. Therefore, a daily oral dose of 600 mg may be reasonable for a study with patients suffering from biliary tract cancer. Drug-related adverse effects of imatinib mesilate include nausea, vomiting, myalgias, edema, diarrhea, and, less commonly, anemia, thrombocytopenia, neutropenia, and myelosuppression[43-45]. Moreover, there are concerns regarding the drugs liver toxicity in biliary tract cancer patients since it is primarily metabolized in the liver and tumor infiltration of the liver may reduce liver

function. Therefore, Eckel *et al* studied pharmacokinetics of imatinib mesilate in 17 patients with hepatocellular carcinoma and Child A-liver cirrhosis and compared the data with the data of 6 patients with CML and normal liver function[46]. As a result, they found no changes in pharmacokinetics in the first group of patients, thus letting assume the drugs safety in patients with hepatocellular carcinoma and Child A-liver cirrhosis. It is likely that these data can be transferred to patients with invasive biliary tract cancer. However, a phase I/II study is now required to further evaluate our preliminary in vitro and in vivo data. Therefore, our group has designed a clinical study for patients with biliary tract cancer that is now in progress.

In conclusion, *c-kit* expression is detectable by immunoblotting, immunocytochemistry, and immunohistochemistry at a moderate to low protein level in biliary tract cancer. Imatinib mesilate exerts marked effects on tumor growth *in vitro* and *in vivo* dependent on the level of *c-kit* expression. A phase I/II study has been designed to further study the role of imatinib mesilate treatment in patients with biliary tract cancer.

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CLINICAL RESEARCH

Effect of 5-HT$_1$ agonist (sumatriptan) on anorectal function in irritable bowel syndrome patients

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Abstract

AIM: To evaluate the effect of sumatriptan, a selective 5-HT$_1$ agonist, on anorectal function in irritable bowel syndrome (IBS) patients.

METHODS: Twenty-two IBS patients selected according to the Rome II criteria (F 15, M 7; mean age 29.3±6.8, range 22-44 years) were examined. The study was blind, randomized and placebo-controlled with a crossover design. Anorectal manometry and rectal balloon distension test were performed before and after the administration of placebo and sumatriptan.

RESULTS: The administration of sumatriptan caused a significant increase in the resting anal canal pressure from 9.2±2.0 kPa to 13.1±3.3 kPa (P<0.0001) connected with the increase in the anal sphincter length and high pressure zone. After sumatriptan injection a remarkable increase in the threshold for the first sensation from 27±9 mL to 34±12 mL (P<0.05) and urge sensation from 61±19 mL to 68±18 mL (P<0.01) was observed. Sumatriptan did not affect either the volume evoking the rectoanal inhibitory reflex or the results of the straining test.

CONCLUSION: 5-HT$_1$ receptors participate in the regulation of anorectal function. Elucidation of the role of 5-HT$_1$ receptors in the pathophysiological mechanisms of IBS may have some therapeutic implications.

Key words: Sumatriptan; 5-HT receptors; Irritable bowel syndrome, Anorectal function

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is one of the main neurotransmitters involved in the control of the gastrointestinal tract function and plays an important role in the pathophysiology of irritable bowel syndrome (IBS)[1,2]. A number of abnormal motor and sensory patterns reported in IBS patients is supposed to be a result of disturbances in serotonergic mechanisms. Different 5-HT receptor subtypes are involved in the regulation of enteric reflexes and signaling to the central nervous system[3]. Pharmacological data confirm that the specific 5-HT$_3$ antagonists and 5-HT$_4$ agonists are beneficial in the management of IBS[4-6]. Among new emerging serotonergic agents for functional gastrointestinal disorders the role of 5-HT$_1$ agonists was discussed[7]. Many IBS patients complain of various non-gastrointestinal symptoms and disorders such as headache, migraine, non-cardiac chest pain, back pain, dysuria, sleeping difficulties, chronic fatigue syndrome, fibromyalgia syndrome, anxiety and depression. From the clinical point of view frequent coexistence of IBS with headache reported by 23%-45% of IBS patients may also indicate the common pathogenetic and therapeutic link for these diseases, possibly related to the serotonin-dependent processes[8].

Sumatriptan is a selective 5-HT$_{1B/D}$ agonist used for migraine treatment. Recent research results on sumatriptan effects on the gastrointestinal tract function have shown that the drug alters oesophageal motility, favours transient lower oesophageal sphincter relaxations despite the increase in the lower oesophageal sphincter pressure, prolongs postprandial fundic relaxation delaying gastric emptying, and induces a premature intestinal third phase of the migrating motor complex in the jejunum. Encouraging results have been obtained in patients with dyspepsia and impaired postprandial gastric relaxation treated with sumatriptan[9,10]. A therapeutic potential for 5-HT$_1$ agonists in IBS patients has been also suggested according to the observation that sumatriptan causes a significant relaxation of the descending colon influencing the perception of colonic distension.

Anorectal function in IBS draws attention due to commonly reported symptoms by patients with this disorder including urgency, excessive straining or feeling of incomplete evacuation. Up to now, consistent association

between the above symptoms and anorectal dysfunction has not been clearly established. In some IBS patients anorectal manometry is performed to exclude abnormalities that may coexist with or imitate IBS. Moreover, assessment of anorectal function in IBS patients, particularly by use of anorectal manometry, presents an easily accessible way to evaluate the effects of drugs on the lower gastrointestinal tract function[11].

The aim of the present study was to test the hypothesis that sumatriptan influences anorectal function in patients with IBS.

MATERIALS AND METHODS

Subjects

Twenty-two IBS patients selected according to the Rome II criteria (15 women (F), 7 men (M); mean age 29.3± 6.8, range 22-44 years) participated in the study. Based on the bowel pattern, three subgroups of IBS patients were distinguished – constipation predominant IBS (C-IBS: 5 F, 0 M), diarrhea predominant IBS (D-IBS: 3 F, 4 M), and alternating IBS (A-IBS: 7 F, 3 M).

The organic causes for the symptoms were excluded by the evaluation of detailed medical history and physical examination, basic laboratory tests and normal colonoscopy within the five years preceding inclusion in the study. Patients who previously underwent abdominal or gastrointestinal surgery, except for appendectomy, were not included. Patients with ischemic heart disease, arterial hypertension, after myocardial infarction or cerebral stroke, and over 45 years of age were not included in the study, either. Blood pressure and heart rate values in all subjects were within the normal range. All patients had a normal electrocardiogram performed just before the study. The study was approved by the Ethics Committee of the Medical University Hospital and written informed consent was obtained from all subjects.

Recording methods

Anorectal manometry and rectal balloon distension were performed using a four lumen water-perfused catheter with a polyethylene balloon attached to the tip (Zinetics Manometric Catheter, Medtronic). Pressure was sensed by external pressure transducer connected to an analogue-digital converter (PC Polygraph, Synectics Medical, Synecpol). The system was calibrated at 0 and 6.67 kPa at the beginning of each study. The following measurements were derived from the manomertic recordings: the maximal anal resting pressure (MRP) and the maximal anal squeeze pressure (MSP); the anal sphincter length (SL) and high pressure zone (HPZ) within the anal canal, both during rest and squeeze. Moreover, strain, squeeze and cough tests as well as volume initiating the internal anal sphincter relaxation (rectoanal inhibitory reflex, RAIR) and volume required to elicit first sensation, urgency and discomfort or pain were evaluated.

The maximal resting and squeeze pressure along the anal canal was measured from the rectum to the anal margin by the dynamic pull-through technique. The procedure was performed both in the normal relaxed state and in the voluntary squeeze state by pulling a catheter through the sphincters at a speed of 10 mm/s. Three pulls in both states were performed. Evaluation of the strain and cough tests were carried out using the stationary pull-through technique. RAIR and sensory thresholds were measured by the intermittent, phasic rectal distension, starting with 10 mL of air and increasing in steps of 10 mL. The rectal balloon was emptied after each distension. The duration of the distension amounted to 60 s, with 40-60 s intervals between each inflation. During distensions subjects were asked to report and classify their sensation in terms of the first sensation, urge sensation and the maximal tolerable volume due to discomfort or pain. Whenever unbearable discomfort or painful sensation were experienced during any level of distension the experiment was immediately suspended.

Study protocol

Patients examined after an overnight fast without a cleansing enema in the left lateral position were allowed to acclimate to the assembly for 20 min. Patients were instructed not to take any analgesic, spasmolytic, anxiolytic, antidepressant, or anti-inflammatory agents within 72 h before the examination. Women participating in the study were examined within the first part of the menstrual cycle. The study was blind, randomized and placebo-controlled with a crossover design, in which each subject received placebo and drug on two separate days at least one week apart. After the baseline recordings sumatriptan 6 mg sc (Imigran, Glaxo Wellcome Group) or saline 0.5 mL sc was given in a random fashion. The second recordings were performed 30 min after the administration of the drug or placebo. Therefore, four recordings in each subject were performed (recording before and after sumatriptan injection, and recording before and after placebo injection). Blood pressure and heart rate were monitored during the study. All adverse events were noted.

Pressure data converted by PC polygraph were displayed, recorded, and analyzed on an IBM-compatible computer using Polygram software (Synectics Medical). Baselines were automatically set by the computer program. The amplitude was measured from the baseline to the peak of the resting and squeeze pressure area. The maximal resting anal pressure and the maximal squeeze anal pressure were defined as the mean of the three highest values observed at any site in the anal canal during three pulls in the relaxed state, and in the squeeze state, respectively. Each increase in the pressure during pull was later marked as an event in the tracing. The position of the event marked the position of the anal sphincter. The anal sphincter length is a product of the applied catheter pulling speed and time of the pressure increase during the pull-through procedure. The length of the zone with a pressure higher than the half of the maximal anal resting pressure value was defined as the high pressure zone.

The cough reflex was evaluated by measuring the mean of the three highest increments in the anal canal pressure during cough. The anal canal pressure during attempts to defecate was evaluated to assess the presence of pelvic floor dysynergia. RAIR was defined as a 20% reduction or more in the resting anal pressure in response to the rectal distension. The perception corresponding to each distend-

Table 1 Effect of sumatriptan on manometric and volumetric anorectal parameters in IBS patients (mean ± SD)

Anorectal parameter	Placebo	Sumatriptan
Maximal resting pressure	9.2±2.0 kPa	13.1±3.3 kPa[b]
Sphincter length during rest	4.0±0.5 cm	4.5±0.4 cm[b]
High pressure zone during rest	2.6±0.3 cm	3.1±0.5 cm[b]
Sphincter length during squeeze	5.1±0.4 cm	5.4±0.5 cm[b]
Urge sensation threshold	61±19 mL	68±18 mL[b]
First sensation threshold	27±9 mL	34±12 mL[c]
High pressure zone during squeeze	2.8±0.6 cm	3.0±0.7 cm[c]
Pain threshold: ≤100mL/>100mL (number of patients)	13/9	8/14
Rectoanal inhibitory reflex (RAIR)	21±8 mL	21±9 mL
Straining test results:		
Proper relaxation/lack of relaxation/ contraction (number of patients)	14/7/1	13/8/1
Maximal squeeze pressure	25.5±8.5 kPa	27.2±8.4 kPa
Maximal cough pressure/maximal resting pressure	2.9±0.8	3.1±1.1

[b]P<0.01, [c]P<0.05, *vs* Placebo.

Paired Student's t test was used in all cases except for analysis of straining test results and pain thresholds where chi-square test was used.

ing volume up to 100 mL was recorded.

Statistical analysis

Data are expressed as mean±SD unless otherwise stated. Statistical analysis was performed using paired Student's t test and the chi-square test, as well as EPIINFO statistical package ver. 3.2. Differences were taken to be significant for values of P<0.05.

RESULTS

Influence of placebo on anorectal function

The effect of placebo on the anal sphincter function and rectal perception thresholds was assessed in all 22 IBS patients participating in the study. Administration of placebo had no significant influence on the manometric parameters or on visceral perception thresholds. No changes in the distending volumes inducing the first rectal perception, urgency or pain sensations were observed. Lowered pain threshold (distending volume ⩽ 100 mL), considered as a sign of visceral hypersensitivity in IBS patients, was reported before and after the placebo administration in 12 and 13 patients (55% and 59%), respectively.

Effect of sumatriptan on the anal sphincter function

As compared with placebo, sumatriptan considerably increased the maximal resting pressure (MRP) from 9.2±2.0 kPa to 13.1±3.3 kPa (P<0.0001). The rise in the maximal squeeze pressure (MSP) was not statistically significant (P=0.0634, Table 1). After sumatriptan injection the increase in the anal canal pressure in response to cough (maximal cough pressure, MCP) was higher than after placebo injection. However, the increase in the MCP was proportional to the corresponding resting pressure, as the ratio MCP/MRP after the drug injection was not altered (P=0.245, Table 1). Manometric evaluation of the anal canal pressure during attempts to defecate revealed improper response in 9 patients (41%). Signs of pelvic floor dyssynergia appeared as paradoxical contractions of the anal sphincters in one subject and as a failure to relax the pelvic floor in 8 patients. The administration of the drug, as in the placebo case, did not change the results of the straining test. The anal sphincter length as well as high pressure zone of sphincter pressure evaluated by the pull-through technique during both rest and squeeze remarkably increased after sumatriptan injection (Table 1).

In comparison of the volume evoking the rectoanal inhibitory reflex (RAIR) after placebo and sumatriptan administration, which were respectively 21±8 mL and 21±9 mL, no significant difference was shown (P=0.665).

Effect of sumatriptan on visceral perception

According to the conscious rectal sensitivity thresholds, 30 min after sumatriptan injection significant increases in the thresholds for the first sensation from 27±9 mL to 34±12 mL (P< 0.05) and urgency from 61±19 mL to 68±18 mL (P< 0.01) were observed. During the baseline recordings lowered rectal pain sensation evoked with the distending volume up to 100 mL was observed in 13 IBS patients (59%) including 5 out of 7 D-IBS patients, 8 out of 10 A-IBS patients, and none of C-IBS patients. After the administration of the drug rectal pain threshold increased in 5 out of 13 patients, but in 8 (36%) it was still evoked by the distending volume up to 100 mL. The increase in the pain threshold was not significant (P=0.227).

Adverse events

After placebo administration in 5 out of 22 patients (23%) transient burning sensation in the place of injection was reported. No other adverse events were observed, except for one patient who complained of mild but long-lasting headache (about 7 d). A variety of side effects after the drug administration was reported in 20 out the 22 patients enrolled (91%). The majority of the adverse events were mild to moderate in severity. They began at a mean of 4 min (range 2-8 min) after sumatriptan injection and lasted for a mean of 12.5 min (range 1-25 min). The most commonly reported adverse events after sumatriptan injection were: burning sensation in the place of injection in 45% of patients; headache and throat tightness in 36%; weakness in 32%; tingling of lower and upper jaw or temple and formication of the head or face skin in 27%; flare in the place of injection, heaviness in chest, limbs, and in head in 23%; nose tightness, dyspnoea, bodily warmth, acroparesthesia, abdominal pain or discomfort in 9%; and nausea, drowsiness as well as total numbness in 4.5%. Many side effects occurred simultaneously, but the typical triad of symptoms, called "triptan sensations", i.e. throat tightness, heaviness in chest and bodily warmth, was not observed in any patient. In two patients after sumatriptan administration short-lasting proctalgia (about 1 min) occurred.

DISCUSSION

Based on the manometric evaluation, anorectal dysfunction in IBS patients included the signs of pelvic floor dysynergia observed in 41% of patients and lowered visceral pain thresholds in 59% of patients, particularly in

those with diarhoea predominant IBS and with alternating bowel pattern. According to the literature data an obstructive pattern of defecation is exhibited in about 20% of healthy subjects. Two times higher prevalence of improper response to the straining test in IBS patients, whether constipated or not, may suggest general changes in pelvic floor mobility in IBS. The presented results have shown that sumatriptan, a selective 5-HT$_{1B/D}$ agonist, noticeably affects anorectal function in IBS patients.

The drug administration in a standard therapeutic dose of 6 mg sc induces a considerable increase in the maximal anal resting pressure, and much less significant increase in the maximal anal squeeze pressure. The increase in the anal canal resting pressure observed after sumatriptan administration reflects mainly the internal anal sphincter contraction. On the contrary, squeeze pressure depends mostly on the external anal sphincter. Similarly, other manometric parameters characterising anorectal function and being connected with the anal canal pressure, such as the functional sphincter length and high pressure zone, increased as well. The maximal cough pressure after the drug injection was proportional to the corresponding resting pressure. Sumatriptan did not have any impact on the straining test results.

Interestingly, sumatriptan has also revealed its influence on visceral sensation thresholds. The increases in the first sensation and urge sensation thresholds were proved to be statistically important. During evaluation of rectal sensation, lowered pain threshold with the distending volume up to 100 mL was evoked in 59% IBS patients, which is in accordance with the literature data[12]. Visceral hypersensitivity was common in D-IBS and A-IBS, but not in C-IBS. After sumatriptan administration a tendency to lower visceral hypersensitivity in IBS patients was observed. The statistical analysis, however, did not confirm the significance of the increase in the pain threshold probably due to methodological limitations as with the maximal distending volume amounting to 100 mL not in all IBS patients pain or discomfort sensation was evoked. To evaluate precisely the influence of sumatriptan on rectal pain threshold another study would be needed, in which higher distending volumes and optimally a barostat or tensostat would be used. Regarding the results of the recent studies which have shown that sumatriptan affects the discomfort threshold during gastric and colonic distension, similar observation may be expected in the rectum. The influence of sumatriptan on colonic motility is important for further evaluation.

For better understanding of the role of 5-HT$_1$ receptors, the effect of sumatriptan on lower gastrointestinal tract function in the different subgroups of IBS patients as well as in healthy subjects should be investigated. From the methodological point of view, a crossover design of the study enabled us to avoid considerable interindividual variability common in functional testing such as anorectal manometry. However, an intraindividual variability could still have some impact on the manometric and volumetric parameters. Foster and colleagues used a similar protocol of the study evaluating the effect of sumatriptan on the oesophageal motility. All the women participating in the study were investigated in the follicular phase, as it has been shown that the menstrual cycle affects rectal sensitivity in IBS patients but not healthy volunteers[13]. Patients were examined after an overnight fast to eliminate the gastrointestinal reflexes and the postprandial increase in visceral sensitivity[14,15].

The majority of the adverse events were mild to moderate in severity and short-lasting. However, it cannot be totally excluded that they might stress the patients, and in this way affect the results to some extent.

Several recent studies dealt with the effect of sumatriptan on the upper part of the gastrointestinal tract[9]. Two observations were particularly important from the clinical point of view. The first one concerned chest symptoms, which occurred in 3-5% of patients using sumatriptan in the migraine treatment. Foster *et al* in the study on 16 healthy subjects have shown that a therapeutic dose of sumatriptan (6 mg, sc) altered oesophageal motility without affecting the ECG, supporting at the same time an oesophageal rather than cardiac cause for the sumatriptan-induced chest pain. However, contrary to what happened in healthy subjects, the drug failed to modify either the wave's amplitude or lower oesophageal sphincter tone in patients with ineffective oesophageal motility[16]. The second important observation having a potential therapeutic implication concerned the influence of sumatriptan on gastric fundic tone and sensitivity to distension[9]. Tack and co-workers[9] have shown that in some dyspeptic patients sumatriptan is able to restore impaired gastric accommodation to a meal and improve the early satiety symptom. A therapeutic potential for 5-HT$_1$ receptor agonist is suggested not only in functional dyspepsia, but in other functional gastrointestinal disorders, in particular IBS. The preliminary observation has revealed that sumatriptan causes a significant relaxation of the descending colon influencing the perception of colonic distension.

The present study has confirmed that sumatriptan affects not only the upper but also the lower part of the gastrointestinal tract, including anorectal function and rectal sensation thresholds. The sumatriptan-induced decrease in rectal sensitivity may have a therapeutic implication in IBS patients, particularly in patients with visceral hypersensitivity.

The effect of sumatriptan on the anal sphincter function should be also considered in patients using this drug in the form of suppository. In two patients after sumatriptan injection a short-lasting, transient proctalgia occurred. Hypothetically, it could result from the increase in the maximal resting pressure, or even spasm of the anal sphincter. However, no clinical observations have confirmed this hypothesis so far. On the other hand, various forms of the drug may differently affect gastrointestinal function. For example, it has been shown that, unlike the subcutaneous formulation, the intranasal administration of sumatriptan has no significant effect on gastric sensory and motor function, probably due to a low bioavailability of intranasally administered sumatriptan[17].

Since the resting anal canal pressure depends essentially on the internal anal sphincter (IAS) function the increase in its value after sumatriptan administration seems to result mainly from the IAS contraction. Up to now, research on neurological and pharmacological control of the IAS

function has evaluated quite precisely the role of the adrenergic and cholinergic systems. Recently, the role of non-adrenergic non-cholinergic (NANC) system involving nitric oxide (NO) as a neurotransmitter has been also investigated. Anorectal manometry and rectal sensitivity testing proved to be a useful method for evaluating the influence of pharmacological agents including serotonergic drugs acting on anorectal function in IBS patients[11]. It has been shown that serotonin induces contraction of the IAS, while ketanserin (a 5-HT_2 receptor antagonist) and cisapride (a 5-HT_4 receptor agonist and partial 5-HT_3 receptor antagonist) evokes the IAS relaxation in healthy subjects[18,19]. Prucalopride, a novel selective 5-HT_4 receptor agonist, seems not to influence the anorectal function either in IBS patients or in healthy controls[20], whereas tegaserod, the next 5-HT_4 receptor agonist, decreases sensitivity to rectal distension in healthy subjects[21]. In the study of Thumshirn *et al*[22] alosetron, a 5-HT_3 receptor antagonist, has no significant effect on gastrointestinal transit or rectal sensory and motor mechanisms in patients with non-constipated IBS. Interesting results have been obtained by Siproudhis *et al*[23] who investigated effects of two types of serotonergic antidepressants, amitriptyline and fluoxetine, on anorectal motility and visceral perception. Both antidepressants similarly relaxed the IAS, probably through a non-specific mechanism, without modifying visceral perception. Only amitriptyline relaxed the external anal sphincter[23].

The mechanisms underlying the sumatriptan-induced anorectal function changes remain unclear. The distribution of various types of 5-HT_1 receptors and particularly their role in the gastrointestinal function are also poorly identified. Anorectal function, and in particular visceral perception, are modulated at different levels of the brain-gut axis and theoretically, sumatriptan could be acting at each of these levels[24]. Potential mechanism for the effect of sumatriptan on anorectal function could occur via activation of the 5-$HT_{1B/D}$ receptors acting on enteric neurons. Alternatively, sumatriptan could be acting on sensory nerve terminals to modulate neurotransmitter release. A further possible mechanism could involve a central action of sumatriptan. However, pre-clinical data indicate that sumatriptan only poorly penetrates the blood-brain barrier making a central mechanism less probable.

A nitrergic pathway as a possible mechanism of the drug action on the gastrointestinal function has also been discussed. It has been already shown that the sumatriptan-induced relaxation of gastric fundus is partially mediated through the activation of an NANC mechanism, involving NO as a neurotransmitter, and the sumatriptan-induced relaxation of the gastric fundus is reversibly blocked by inhibition of NO synthase. However, regarding the fact that NO is the main neurotransmitter involved in the occurrence of RAIR, the lack of sumatriptan effect on the volume evoking RAIR might argue against the nitrergic mechanism. More likely a direct smooth muscle response may be modified. Sumatriptan induces not only the anal sphincter contraction, but as it has been shown in another study it causes also the increase in the lower oesophageal sphincter pressure.

Furthermore, it has been already shown that sumatriptan is able to induce endocrine secretion in men, and at the same time, for example somatostatin or glucacon can affect anorectal function. However, measurement of plasma somatostatin and glucagon concentration before and after administration of sumatriptan rules out their release as a mechanism by which sumatriptan may influence gastrointestinal function.

Sumatriptan may alter the perception of rectal distension due to its direct impact on the rectal tone. It has been already shown that the sumatriptan-induced gastric or colonic relaxations induce changes in visceral perception[9,10]. Likewise, higher volume thresholds during rectal sensitivity testing after sumatriptan administration may occur secondary to the drug-induced relaxation of the rectum. Therefore, further studies using a barostat or impedance planimetry are needed to explain this issue. The results obtained hitherto warrant further studies to clarify and verify the regulatory role of 5-HT_1 receptors in the gastrointestinal function and the mechanisms responsible for the effect of sumatriptan on the gastrointestinal sensorimotor function including receptor subtypes involved, and central *vs* peripheral mechanism. Selective and safe 5-HT_1 receptor ligands which are now lacking will be crucial for the future research.

In conclusion, the effect of sumatriptan on the anal sphincter function and rectal sensitivity thresholds indicates that 5-HT_1 receptors participate in the regulation of anorectal function. Better understanding of the role of these receptors in the pathogenesis of IBS may have some therapeutic implications, particularly in patients with visceral hypersensitivity. The possible application of anti-migraine drugs in the management of functional gastrointestinal disorders remains an open issue.

Preliminary results of this study have been presented in part at the 12th United European Gastroenterology Week held in Prague, Czech Republic, September 2004, and published as an abstract in *Gut* 2004; 53 (Suppl VI): A204.

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CLINICAL RESEARCH

Effect of gemcitabine on the expression of apoptosis-related genes in human pancreatic cancer cells

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Abstract

AIM: To investigate the expression of genes involved in the gemcitabine-induced cytotoxicity in human pancreatic cancer cells.

METHODS: A human pancreatic cancer cell line, PANC-1, was cultured. 1 x 10^4 PANC-1 cells were plated in 96-well microtiter plates. After being incubated for 24 h, gemcitabine was added to the medium at concentrations ranging 2.5 -1 000 mg/L. The AlamarBlue dye method was used for cell growth analysis. DNA fragmentation was quantitatively assayed using a DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit. *PAP* and *TP53INP1* mRNA expression was determined using the reverse transcription-polymerase chain reaction with semi-quantitative analysis. The expression of GSK-3β and phospho-GSK-3β proteins was examined with Western blot analysis.

RESULTS: The IC_{50} for the drug after a 48-h exposure to gemcitabine was 16 mg/L. The growth of PANC-1 cells was inhibited by gemcitabine in a concentration-dependent manner ($P < 0.0001$) and the cell growth was also inhibited throughout the time course ($P < 0.0001$). The DNA fragmentation rate in the gemcitabine-treated group at 48 h was 44.7 %, whereas it was 25.3 % in the untreated group. The *PAP* mRNA expression was decreased after being treated with gemcitabine, whereas the *TP53INP1* mRNA was increased by the gemcitabine treatment. Western blot analysis showed that phospho-GSK-3β^{ser9} was induced by the gemcitabine treatment.

CONCLUSION: Gemcitabine suppresses PANC-1 cell proliferation and induces apoptosis. Apoptosis is considered to be associated with the inhibition of PAP and GSK-3β, and the activation of *TP53INP1* and pospho-GSK-3β^{ser9}.

Key words: Gemcitabine; Pancreatitis-associated protein; TP53INP1; GSK-3β; PANC-1

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INTRODUCTION

Pancreatic cancer is the fifth most common cause of cancer death in Japan[1]. It is estimated that there are 20000 cases every year, which is similar to the number of deaths from this disease. The reasons for its very high mortality rate include the lack of early diagnosis, a low resectability at the time of initial diagnosis, and the rapid recurrence after resection. Surgery is rarely a curative option in pancreatic cancers because of local extension and metastasis. Chemotherapy for advanced pancreatic cancer is palliative. The use of 5-FU in combination with radiation in the locally advanced setting has been shown to enhance survival. In a randomized trial between gemcitabine and 5-FU, gemcitabine showed significantly better results in terms of the clinical benefit effect and survival[2].

Gemcitabine (2', 2'-difluorodeoxycitidine) is a nucleoside analogue well known for its antitumor activity in several solid tumors[3]. It is one of the drugs effective for pancreatic cancers in the clinical treatment[4]. Various general mechanisms of gemcitabine have been described[5-7]. A primary mechanism of gemcitabine is the blocking of DNA synthesis by inhibiting DNA polymerase activity[8]. Gemcitabine itself is not active until it enters the cells. Its intracellular transport is mediated by facilitated diffusion[9]. Intracellularly, gemcitabine is phosphorylated to its active metabolites by deoxycitidine kinase to difluorodeoxycitidine monophosphate (dfdCMP), difluorodeoxycitidine diphosphate (dfdCDP) and difluorodeoxycitidine triphosphate (dfdCTP). All three of the metabolites interfere at different steps in the processing of DNA. The dfdCTP is incorporated into

DNA and as such can obstruct DNA replication and repair. The dfdCTP can also be incorporated into RNA and can inhibit CTP-synthetase. The dfdCMP can also inhibit dCMP-deaminase. The dfdCDP is an inhibitor of ribonucleotide reductase, and its action was shown to lead to the depletion of the DNA precursor pool, dNTP. Inhibition of DNA synthesis leads to growth inhibition or cell death. In previous studies, gemcitabine has been shown to play a major role in the apoptosis of certain tumor cell lines[10-12]. There are several pathways that relate to gemcitabine-induced apoptosis. Nicole *et al*[13] reported that gemcitabine-mediated apoptosis is caspase-dependent in pancreatic cancers; Jones *et al*[14] showed that gemcitabine-induced apoptosis is achieved through the blocking of NF-kB in non-small cell lung cancer cells (NSCLC). In addition, gemcitabine-induced apoptosis is also related to signal-regulated kinase (ERK), Akt, Bcl-2 and p38 mitogen-activated protein kinase (MAPK) pathways[15-17]. Achiwa *et al*[18] indicated that the increase in human equilibrate nucleoside transport (hENT) expression is a determinant of gemcitabine sensitivity in NSCLC cells. However, the molecular mechanism of gemcitabine-induced apoptosis has not been fully elucidated.

Pancreatitis-associated protein (PAP) is a secretory protein of pancreatic acinar cells. It is almost absent in the normal pancreas, but is induced in acute and chronic pancreatitis. We have reported the expression of *PAP* mRNA in cancer tissues and have measured *PAP* levels in the sera and pancreatic juice of patients with gastrointestinal cancers[19-21]. We found that serum *PAP* levels were increased in 40% of patients with pancreatic cancer. We also reported that *PAP* levels in endoscopically aspirated pancreatic juice were positive in 55% of pancreatic cancers. *PAP* levels were significantly higher in both the serum and pancreatic juice in cases of pancreatic cancer, compared to chronic pancreatitis. Cytokines such as tumor necrosis factor-α, interferon-γ, and interleukin-6 induce *PAP* mRNA expression in the pancreatic acinar AR4-2J cell line. We found that the enhanced expression of *PAP* in pancreatic adenocarcinoma is caused by both ectopic expression in cancer cells and induction in acinar cells[22].

TP53INP1[23] was previously called stress-induced protein (SIP)[24] or p53-dependent inducible nuclear protein (p53DINP1)[25]. *TP53INP1* is strongly induced in acinar cells during acute pancreatitis in mice, and is also overexpressed in response to various stresses in vitro. *TP53INP1* gene expression is wild-type p53-dependent[26]. There is a functional p53-response element within the promoter region of the *TP53INP1* gene, and *TP53INP1* mRNA expression is activated in cells expressing wild-type p53 in response to various stresses. One of the major functions of TP53INP1 is promoting cellular apoptosis. Glycogen synthase kinase 3β (GSK-3β) is a multifunctional serine/threonine kinase mediating various cellular signaling pathways. The particular pathway depends on its substrates for phosphorylation[27]. Since GSK-3β is also an important mediator of an apoptotic signal, it is plausible that the GSK-3β deregulation observed in cancer cells confers resistance to chemotherapy, which is a major cause of treatment failure in human cancers[28].

In this study we investigated the effect of gemcitabine on the PANC-1 cells in terms of apoptosis-related factors.

MATERIALS AND METHODS

Cell culture and gemcitabine treatment

A human pancreatic cancer cell line, PANC-1, obtained from the American Type Culture Collection (ATCC, MD, USA), was maintained in Dulbecco's modified Eagle's medium supplemented with 100 mL/L fetal calf serum, penicillin, and kanamycin at 37℃ in a 50 mL/L CO_2, 950 mL/L air atmosphere. Gemcitabine (Eli-Lilly Japan, Kobe, Japan) at a concentration of 50 mg/mL was dissolved in the serum free culture medium and stored at -20℃ in the freezer. The concentration range of the treatment was from 2.5 mg /L to 1 000 mg/L.

Cell growth evaluation

The Alamarblue dye method was used for cell growth analysis. The 1×10^4 cells were plated in 96-well microtiter plates. After being incubated for 24 h, gemcitabine was added to the medium. Twenty μL of AlamarBlue dye solution (Iwaki Glassware, Inc., Tokyo, Japan) was added to wells containing 200 μL of medium at the time 12, 24, 48, and 72 h. After being incubated for 3 h, the cell growth was evaluated as the absorbance (A) using a spectrophotometer (Dai-Nippon Pharmaceutical Co., Osaka, Japan). An excitation wavelength of 540 nm was used, and the emission was read at 620 nm. The color of AlamarBlue stock is violet, and changes to red when oxidized. Each treatment was applied to 6 wells, and the experiments were repeated 3 times.

DNA fragmentation assay

DNA fragmentation was quantitatively assayed using a DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the protocol. Cells were cultured in flat-bottom, 96-well microplates. After incubation in gemcitabine-supplemented media for 24 h, the cells were detached from the wells. The cells were lysed with lysis buffer, and the lysate was processed for streptavidin-coated microtiter plates. After incubation with biotin-labeled antihistone antibody and peroxidase-labeled anti-DNA antibody, the amount of fragmented DNA was determined using 2, 2'-azino-bis-3-ethylbenzthiazoline-sulfonate (ABTS) as a substrate. The plates were read on a Labsystems integrated EIA (Dai-Nippon Pharmaceutical Co., Osaka, Japan) at wavelengths of 540 nm and 620 nm. Each treatment was added to duplicate wells, and the experiment repeated 3 times.

Total RNA was extracted from pancreatic cancer cells using a SV Total RNA Isolation system kit (Promega, Madison, WI, USA). RNA concentrations were determined with spectrophotometry. RT was performed using a PowerScript™ Reverse Transcriptase kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). First strand cDNA was synthesized from 8 μg of total RNA at 65 ℃ for 5 min after RT; the reverse transcriptase was inactivated by incubating at 42℃ for 60 min and the reaction was terminated by heating at 70℃ for 25 min. Then, cycles

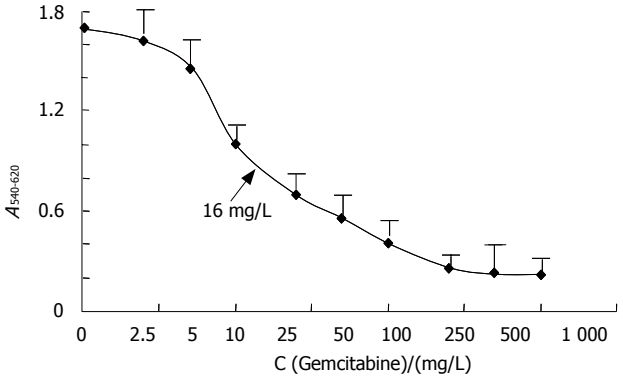

Figure 1 Effect of gemcitabine on the growth of PANC-1 cells.

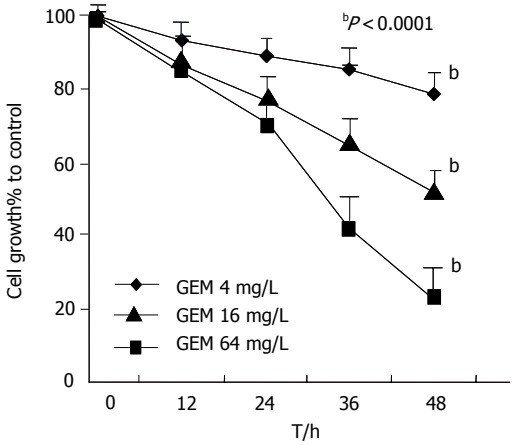

Figure 2 Inhibitory effect of gemcitabine on the growth of PANC-1 cells.

of amplification were performed on a DNA thermal cycler (Perkin Elmer Cetus, Inc., Norwalk, CT, USA) as follows: *PAP* primers were denatured at 91℃ for 60 sec, annealed at 54℃ for 60 sec, and polymerized at 72℃ for 45 sec. Then, extension was performed at 72℃ for 10 min. *TP53INP1* primers were denatured at 94℃ for 50 sec, annealed at 62℃ for 40 sec, and polymerized at 72℃ for 50 sec. The extension was performed at 72℃ for 10 min. The *PAP* primer pairs were sense: 5'-CTCCTGATTGCCTCCTCAAG -3' and antisense: 5'-AAACGTACCCTCTCTTTAGG -3', producing a fragment of 441 bp. *TP53INP1* mRNA was specifically amplified with the following primers: sense 5' -CATCCAGCCAAACTCTCAGTC -3' and antisense 5' -GCGACGAAGGCTATTTCTGT -3'. The size of the fragment was 703 base pairs (bp). The *GAPDH* of 452 bp was used as an internal control. Eight-microgram aliquots of the RT-PCR products were subjected to electrophoresis on a 20 g/L agarose gel and visualized with SYBR Gold (Molecular Probes, Inc., Eugene, OR, USA) at a 1:10000 dilution in dimethylsulfoxide, and exposed to ultraviolet 312-nm light.

Semiquantitative RT-PCR analysis

The gene expression of *PAP* and *TP53INP1* was semiquantitatively analyzed with an image analyzer (ATTO Densitograph ver. 3.02, ATTO, Inc., Tokyo, Japan). The relative expression intensity was calculated according to the following formula: PAP and TP53INP1 mRNA in a sample/GAPDH mRNA in a sample.

Western blot analysis

For Western blot analysis, 1×10^6 PANC-1 cells were lysed in a CelLytic™ mammalia tissue lysis/ extraction reagent buffer (Sigma-Aldrich, St. Louis, MO, USA). Aliquots were boiled for 5 minutes with Laemli buffer, resolved on a 125 g/L SDS-PAGE gel and eletrophoretically transferred to nitrocellulose membrane (Amersham, Buckingham, UK). Membranes were blocked for 1 h in TBS containing 50 g/L dry milk and 1 g/L Tween 20. Membranes were then incubated with either anti-rabbit phospho-GSK-3β (ser9) polyclonal antibody (Cell Signaling Technology, Inc. Beverly, MA, USA) or anti-mouse GSK-3β monoclonal antibody (BD Transduction Laboratories, Lexington, KY, USA) for one night at 4℃. After incubation with anti-rabbit or anti-mouse IgG second antibody labeled with peroxidase, the membranes were visualized with an ATTO image analyzer.

Statistical analysis

Experimental results were expressed as the mean ± SE. The difference between the means was evaluated with the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using StatView-5.0 (SAS Institute Inc. Tokyo, Japan).

RESULTS

Gemcitabine inhibited cell growth

The median 50% inhibitory concentration (IC_{50}) of gemcitabine after a 48-h exposure was 16 mg/L (Figure 1). We also measured the effect of 4, 16 and 64 mg/L gemcitabine at 12, 24, 36, and 48 h. The growth of PANC-1 cells was inhibited by gemcitabine in a concentration-dependent manner ($P < 0.0001$, Figure 2) and cell growth was also inhibited throughout the time course ($P < 0.0001$).

Gemcitabine induced apoptosis

1×10^4 PANC-1 cells were cultured in flat-bottom, 96-well microplates. After incubation in gemcitabine-supplemented media for 24 h, a DNA fragmentation assay was performed. The concentration of gemcitabine used in the DNA fragmentation assay was 16 mg/L. The DNA fragmentation rate in the gemcitabine-treated group was 44.7%, whereas the fragmentation rate in the untreated group was 25.3%. The fragmentation in the gemcitabine treated group was significantly higher than that in the untreated group ($P = 0.0157$).

Expression of PAP, TP53INP1 mRNA

We exposed the PANC-1 cells to 4, 16 and 64 mg/L gemcitabine for 48 h. The expression of *PAP* and *TP53INP1* mRNA was assessed using RT-PCR. The *PAP* mRNA expression was decreased after being treated with gemcitabine, and disappeared after being treated with 16 mg/L gemcitabine. The semiquantitative analysis showed the intensity of 16 mg/L gemcitabine-treated

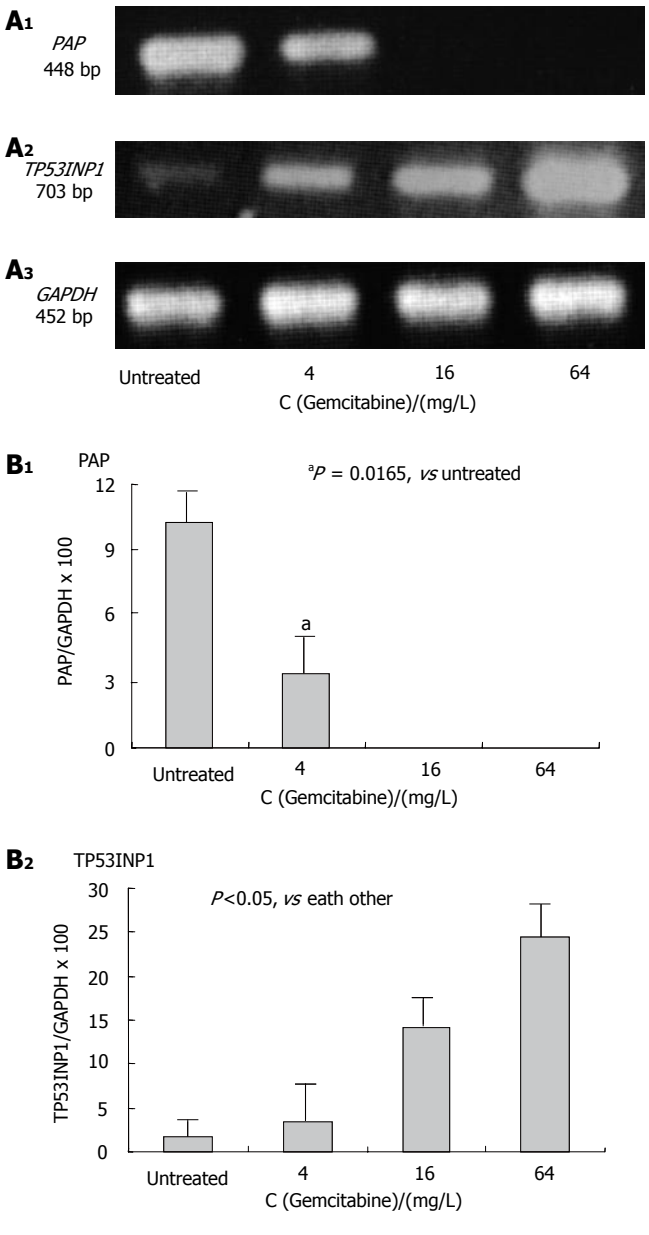

Figure 3 *PAP* mRNA expression after gemcitabine treatment.

cells to be significantly lower than that of the untreated cells ($P = 0.0165$, Figure 3). The *TP53INP1* mRNA was increased after exposure to gemcitabine. The intensity of the cells treated with 4, 16 and 64 mg/L gemcitabine was 2 times, 8.5 times and 14.6 times higher than in the untreated cells, respectively. The increasing density of *TP53INP1* mRNA was concentration-dependent. The *P* value of the interrelation of the untreated group to the 4 mg/L gemcitabine-treated group, 16 mg/L gemcitabine-treated group and 64mg/L gemcitabine-treated group were 0.0015, 0.0009 and 0.0352, respectively.

Expression of phospho-GSK-3β and GSK-3β proteins

The PANC-1 cell line showed no detectable phospho-GSK-3β^{ser9} in Western blotting analysis with a phospho-specific antibody, indicating GSK-3β activity in this cell line (Figure 4). On the other hand, phospho- GSK-3β^{ser9} was induced after 4 mg/L gemcitabine treatment, and gradually increased with the 16 and 64 mg/L gemcitabine

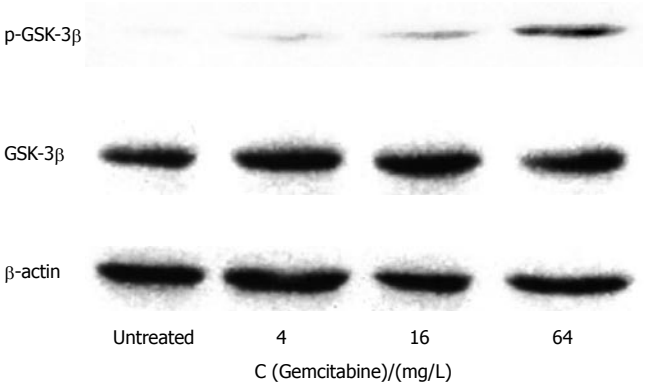

Figure 4 Effect of gemcitabine treatment on GSK-3β expression.

treatment. There was no significant difference in GSK-3β expression between the gemcitabine-treated and untreated groups.

DISCUSSION

Gemcitabine is a nucleotide analog that is converted to its triphosphate active form in cells and is subsequently incorporated into DNA to terminate strand elongation[13-17]. It shows a favorable clinical outcome in the treatment of pancreatic cancer and non-small cell lung cancer[14,18]. However, there have been few published *in vitro* analyses about gemcitabine. We therefore attempted to investigate the genes involved in the gemcitabine-induced cytotoxicity of pancreatic cancer cells. In the present study we showed that gemcitabine induced changes in expression of apoptosis-related genes in PANC-1 cells. The expression of anti-apoptotic pancreatitis-associated protein (PAP) was down-regulated, whereas the pro-apoptotic *TP53INP1* and p-GSK-3β were up-regulated after being treated with gemcitabine.

Apoptosis is believed to play an important role in tumors, and both pro- and anti-apoptotic factors are simultaneously activated in tumor development and progression. In this study, we showed that *PAP* expression was significantly decreased in the gemcitabine-induced anti-tumor process. The growth of human pancreatic cancer may require the up-regulation of *PAP* expression, which would suppress the apoptosis of cancer cells[19]. *PAP* is one of the effectors of antiapoptosis induced by tumor necrosis factor-α through NF-κB and MAP kinases in pancreatic acinar cells[29]. It was demonstrated that the activation status of the NF-κB, Akt and MAP kinase signaling pathways is often associated with anti-apoptotic signal transduction that is linked to chemotherapeutic agents[16]. NF-κB plays an important role in oncogenesis and promotes cellular resistance to anticancer therapy. Bandala *et al*[30] showed that gemcitabine treatment increased the activity of NF-κB, and Alexader *et al*[31] showed that the induction of NF-κB by gemcitabine was dose-dependent in five pancreatic cancer cell lines. In addition, gemcitabine specifically activates p38 MAP kinases in the human pancreatic cancer cell lines, PK-1 and PCI43[16]. Taken together, these findings confirmed that *PAP* is involved in the gemcitabine induced-apoptosis in a

dose-dependent manner.

Apoptosis is one of the major consequences of chemotherapy against malignancies. It is now established that the tumor suppressor p53 inhibits cell growth through the activation of cell cycle arrest and apoptosis. *TP53INP1* is a gene that is activated by wild type p53. It cooperates with HIPK2 to promote p53 phosphorylation at ser 46, and then induces apoptosis-inducing protein activation[25, 26]. In our conditions, we showed *TP53INP1* was up-regulated by gemcitabine treatment from 4 mg/L to 64 mg/L, in a dose-dependent manner. *TP53INP1* may be involved in gemcitabine-mediated apoptosis. The precise mechanism of the *TP53INP1* increase after gemcitabine treatment is unclear. It has been shown that phosphorylation of the NH_2-terminal residues of p53 mediates its stabilization and nuclear accumulation after anticancer drug treatment[28].

Beurel *et al*[28] has reported that GSK-3β is hyperphosphorylated on serine 9 in human hepatoma cell lines as well as in human and murine tumoral livers. We examined the impact of gemcitabine on GSK-3β activity in PANC-1 cells by measuring the phosphorylation level of GSK-3β at serine 9 as an indicator of GSK-3β inactivation. GSK-3β can act as a positive or negative physiological regulator of the p53 protein. During endoplasmic reticulum stress, p53 is inhibited through a mechanism involving its phosphorylation at ser 315 and ser 376 by GSK-3β. On the other hand, GSK-3β plays a crucial role in cell survival mediated by nuclear factor-kappaB (NF-κB) signaling. Phospho- GSK-$3\beta^{ser9}$ is an inactive form in normal tissues that suggests the regulation of the balanced expression of the active and inactive forms of this kinase. In our data, phospho- GSK-$3\beta^{ser9}$ was induced by gemcitabine treatment, indicating that GSK-3βgradually became inactive in the gemcitabine-treated cells. Our results are similar to those of Beurel *et al*[28], who showed that GSK-$3\beta^{ser9}$ phosphorylation by lithium treatment on tumor cells conferred resistance to anticancer therapy.

In conclusion, gemcitabine decreases cell proliferation of a human pancreatic cancer cell line, PANC-1, and promotes cellular apoptosis. The antiapoptotic gene, *PAP*, is down-regulated by gemcitabine treatment, whereas the pro-apoptotic *TP53INP1* gene and GSK-$3\beta^{ser9}$ protein are up-regulated. Gemcitabine can induce apoptosis in cancer cells through GSK-3β and *PAP* inhibition, and *TP53INP1* and GSK-$3\beta^{ser9}$ activation.

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CLINICAL RESEARCH

Mangafodipir trisodium-enhanced magnetic resonance imaging for evaluation of pancreatic mass and mass-like lesions

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Abstract

AIM: To investigate the role of mangafodipir trisodium (MnDPDP) in focal pancreatic masses and mass-like lesions by evaluating contrast uptake features of the lesions and pancreatic parenchyma after contrast medium injection.

METHODS: A total of 37 patients with pancreatic mass or mass-like lesions were examined by unenhanced and MnDPDP-enhanced magnetic resonance imaging (MRI).

RESULTS: MRI was obtained 20-40 min after infusion of MnDPDP and homogeneous contrast enhancement was observed in normal pancreas parenchyma. In patients with atrophic pancreas there was no enhancement in pancreatic parenchyma on MnDPDP-enhanced MRI. In 37 patients with 41 pancreatic masses and mass-like lesions, contrast enhancement was observed at 5 lesions on MnDPDP enhanced MRI. Three of these 5 lesions were focal pancreatitis and the other 2 were adenocarcinoma. No contrast enhancement was determined in 36 pancreatic masses and mass-like lesions in 32 patients.

CONCLUSION: MnDPDP contrast-enhanced MRI, especially in cases with no parenchyma atrophy, can distinguish focal pancreatic lesion margins. Information about the function of pancreatic parenchyma can be obtained out of tumor. MnDPDP facilitates staging of pancreatic tumors by detection of metastatic lesions in the liver. In addition, diminished hetergenous uptake of MnDPDP in patients with pancreatitis may be helpful in differential diagnosis.

Key words: MnDPDP; Pancreas; MRI

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INTRODUCTION

Diagnosis of focal pancreatic lesions is a significant challenge for radiologists and surgeons. Focal pancreatic lesions originate from the exocrine (mainly ductal adenocarcinomas) or endocrine part of gland (apudomas), cysts and/or inflammatory lesions. The diagnosis of apudoma is based on the elevated levels of circulating hormones, while the diagnosis of all other focal pancreatic lesions is mainly based on diagnostic imaging[1].

The relation of tumor with its surrounding structures can be described by cross-sectional techniques such as ultrasonography (US), CT and MRI. Ultrasonography has been proved as a first screening mode to depict the panceatic head and duct, the common bile duct and major vessels, but its value is limited by air in the digestive tract and its operator. Contrast enhanced triphasic spiral CT has an accuracy of only 70% for local staging[2]. Surgical procedures are therefore sometimes needed for diagnostic purposes[3,4]. Consequently there is a need for imaging methods with a higher sensitivity and specifity for identifying and staging pancreatic lesions to diminish the number of unnecessary laparotomies.

MnDPDP (Teslascan, Amersham, GE Heralth Care) is an organ-specific paramagnetic contrast agent that has been developed for imaging of the hepatobiliary system. Significant tissue uptake is seen mainly in the liver but much less in the renal cortex, adrenal glands, pancreas and other abdominal organs[5-7]. MnDPDP enables to get images with a higher signal-to-noise (S/N) ratio[8].

In this study, pancreatic mass and mass-like lesions were examined by MRI performed using MnDPDP. The aim of this study was to investigate the role of MnDPDP in focal pancreatic masses and mass-like lesions by evaluating features of their contrast uptake after contrast medium injection.

Table 1 Localization of pancreatic mass and mass-like lesions

	Patients (*n*)	Localization			Lesions (*n*)
		Head	Corpus	Tail	
Pancreas Ca	24	16	5	4	25[1]
Gastrinoma	1			1	1
Lymphoma	1			1	1
Lipoma	1			1	1
Focal pancreatitis	3	2		1	3
Pseudocyst	4	1	4	2	7[2]
Hydatid cyst	1	1			1
Choledochal cyst	1	1			1
Metastase	1		1		1
TOTAL (%)	37	21 (51.2)	10 (24.3)	10 (24.3)	41 (100)

[1]Two masses in a patient.
[2]Two masses in a patient and three masses in the other.

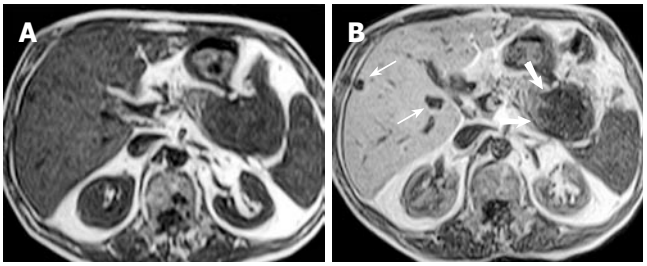

Figure 1 T1-weighted axial SE contrast-unenhanced (A) and MnDPDP-enhanced (B) images of pancreatic adenocarcinoma with liver metastases. MnDPDP enhancement was not determined in the lesion while the normal parenchyma of the pancreas showed homogeneous contrast enhancement. MnDPDP- enhanced image showed the better delination of the tumor (thick arrows). Tiny liver metastases (thin arrows) were not determined on unenhanced MR image but on MnDPDP-enhanced MRI.

MATERIALS AND METHODS

Pancreatic mass lesions or suspicious mass were determined in 37 patients (24 men, 13 women) with a mean age of 58.1 years (range 23-83 years) by US and CT. All patients underwent MnDPDP-enhanced and unenhanced MRI. The patients who received any contrast agent within 1 h before MnDPDP-enhanced MRI or had obstructive hepatobiliary disease or biliary stasis or severe renal impairment, or were contraindicative for CT or MRI or previously enrolled into this study, or defined as pregnancy or lactation, were excluded. The study was approved by the local ethical committee.

MR imaging was performed at 1.5 T (Picker Edge, Picker Internationals Highlands Height, OH) using a body coil. Sequences of axial T2-weighted fast-spin-echo (TR/TE: 8600-9700/96 msec matrix 192×256, slice thickness 3,5 mm, flip angle 90, FOV 40-55 cm), axial and coronal T1-weighted gradient echo (TR/TE: 155/8.1 msec, matrix 160x256, slice thickness 3 mm, gap 0,5 mm, flip angle 60, FOV 40-55 cm, breath-hold 15-20 sec) were applied in all patients before administration of contrast agent. Images of the coronal plane were obtained using only one of the three sequences (SE, gradient echo or fat sat gradient echo).

MnDPDP was injected intravenously at a dosage of 10 μmol/kg (2-3 mL/min) for 15 minutes. Postcontrast imaging was obtained using the same protocol and sequences (except for T2 weighted fast SE sequence) approximately 30 minutes after contrast administration. After contrast medium injection, the size, number, appearance, characteristics of the lesions were compared with normal pancreatic parenchyma. Also contrast uptake level in the lesions and pancreatic parenchyma were evaluated.

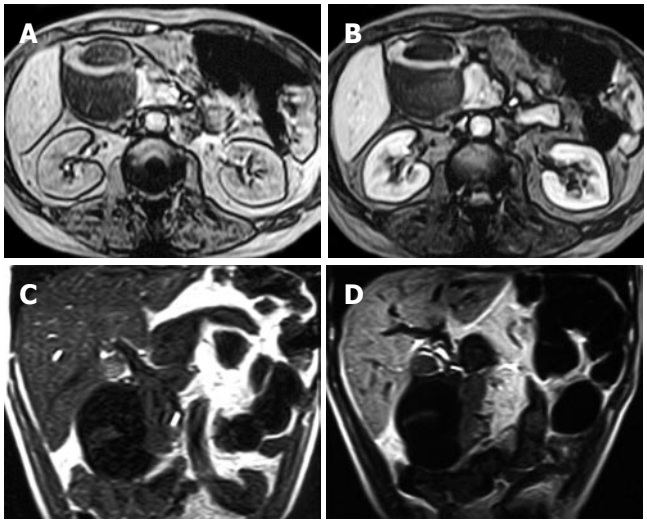

Figure 2 Unenhanced (A) and MnDPDP-enhanced (B) axial T1-weighted GRE images, unenhanced (C) and MnDPDP-enhanced (D) coronal T1-weighted SE images in pancreatic parenchyma out of lesion and liver parenchyma after MnDPDP infusion. Minimal contrast enhancement in the septa of mass was found on MnDPDP-enhanced images (B and D).

RESULTS

Diagnosis of all patients was confirmed by radiological, clinical and histopathological examination (Table 1). Twenty-three of the 37 patients underwent surgical intervention and percutaneous biopsy was performed in 5 patients with primary or metastatic lesions. In the remaining 9 patients, diagnosis was confirmed by clinical and radiological follow-up. Twenty-four patients with pancreatic carcinoma underwent MnDPDP-enhanced abdominal MRI. In 22 of 24 patients, contrast medium uptake in the mass lesions was not determined (Figure 1). Minimal heterogeneous contrast enhancement was seen in one of the 2 patients with contrast-enhanced masses and contrast enhancement of the septa was observed in the other patient with mucinous cystadenocarcinoma (Figure 2).

In patients with pancrea carcinoma contrast enhancement of pancreatic parencyhma out of the mass was evaluatedtic. Contrast enhancement was determined in 13 cases and no contrast enhancement was observed in 11 cases. Homogeneous contrast enhancement of pancreatic parencyhma was seen in 11 cases. MnDPDP-enhanced images revealed a significant improvement in the demarcation and depiction of pancreatic masses as compared to non-enhanced images. Contrast uptake of parenchyma out of mass was minimal in the other 2 cases. In these cases of pancreatic head carcinoma, pancreatitis was found with enlarged and heterogeneous contrast enhancement at the pancreatic parencyhma. In 11 patients with pancreas car-

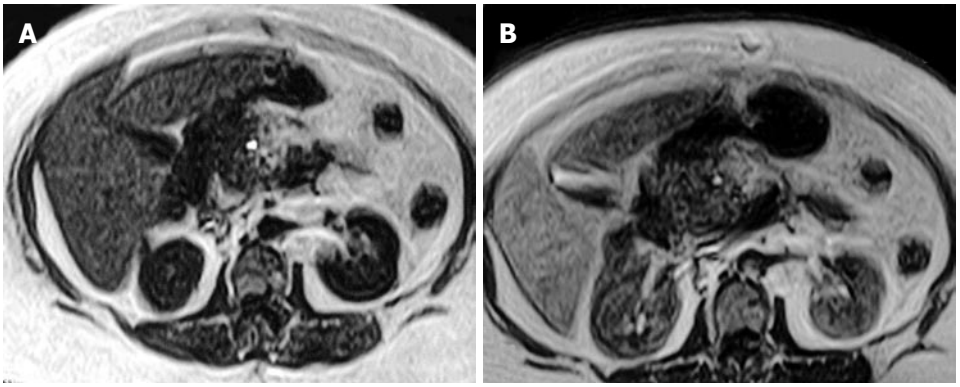

Figure 3 Contrast-unenhanced T1-weighted axial SE image demonstrating focal enlargement of the pancreatic head with a low signal intensity, simulating the appearence of pancreatic cancer (A) and MnDPDP-enhanced T1-weighted axial SE image showing heterogeneous and reduced contrast enhancement in this focal lesion (B).

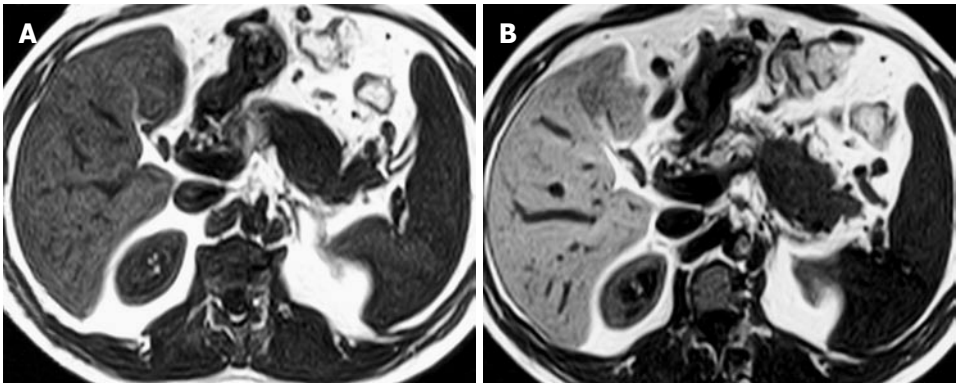

Figure 4 Unenhanced (A) and MnDPDP-enhanced (B) axial T1-weighted SE images showing better delineation of the tumor after MnDPDP infusion.

cinoma, pancreatic parencyhma was atrophic, indicating that these patients after MnDPDP infusion had no contrast enhancement in parencyhma out of the mass. In 3 patients with focal pancreatitis, the lesions were located at the tail in 1 case and at the head in 2 cases. Heterogeneous and reduced contrast enhancement in the focal lesions and homogeneous enhancement of parencyhma out of the lesion were observed in all the 3 cases after MnDPDP infusion (Figure 3). In patients with pancreas pseudocyst, metastatic lesion, hydatid cyst, aggressive type non-Hodgkin's lymphoma, lipoma, choledochal cyst and gastrinoma (Figure 4), no contrast enhancement was seen in all masses after MnDPDP infusion. In 37 patients with pancreatic masses or mass-like lesions, minimal contrast enhancement was established in 5 of 41 lesions (12.2%) by MnDPDP-enhanced MRI. No contrast enhancement was determined in 36 (87.8%) pancreatic masses or mass-like lesions in 32 patients.

DISCUSSION

Ductal adenocarcinoma of the pancreas comprises 75-90 % of all tumors of the pancreas[9]. Abdominal discomfort, back pain, obstructive jaundice and weight loss are the most common symptoms of pancreatic cancer[10]. The only potential cure for pancreatic neoplasms is surgery. Therefore early diagnognosis and assessment of tumor resectability are fundamental to achieve a successful treatment[11]. Bluemke *et al*[3] reported that ductal adenocarcinoma can be detected with helical CT and that further efforts to improve the preoperative staging of pancreatic ductal adenocarcinoma with helical CT should be directed toward improving the detection of small pancreatic ductal adenocarcinomas. Vellet *et al*[12] also reported that non-contour deforming small adenocarcinomas may not be detected by dynamic CT. Small pancreatic masses can be readily detected by MnDPDP-enhanced MRI and the sensitivity of MRI in staging of tumor is increased[13]. Gehl *et al*[14] reported that pancreatic enhancement is increased by 98% after MnDPDP injection. It was also found that after MnDPDP infusion, signal intensity of normal pancreatic parencyhma rises while tumor remains with less signal intensity[15].

Tumor-forming pancreatitis, also known as pseudotumorous pancreatitis or inflammatory pancreatic mass, is presented as a mass-like glandular enlargement. Focal enlargement of the pancreas typically involving head of the pancreas and common bile duct may occur, simulating the development of malignant neoplasms. Since the signal intensity and nonspecific contrast enhancement patterns of this tumor are similar to those of malignant tumor, their differential diagnosis is usually diffucult[16]. In our study, minimal heterogeneous contrast enhancement was observed in 3 patients with local pancreatitis by MnDPDP-enhanced MRI.

In patients with pancreatic adenocarcinomas, the contrast enhancement was compared to the proximal and distal part of tumor after MnDPDP infusion and less enhancement was found in the distal part of tumor. This may be caused by the obstruction of the pancreatic duct which is often found adjacent to the distal part of tumor[1]. In our study, pancreatic contrast enhancement at the distal part of tumor was poor in 2 cases because of pancreatitis adjacent to the distal part of tumor. Duct dilatation and atrophy of the pancreatic tail and body can be seen due to obstruction of the pancreatic duct in malignant tumor at the head of the pancreas. The parenchyma may have

a poor function even though atrophy does not occur, contrast enhancement of parenchyma becomes markedly less[8].

In our study, no uptake of contrast medium was observed in 11 patients with pancreatic malignant tumor by MnDPDP-enhanced MRI. Contrast enhancement of tumor was not observed in patients with tumor (out of 2 cases). On MnDPDP-enhanced T1-weighted images, minimal contrast-enhanced mass was detected in 1 patient with adenocarcinoma at the head of pancreas and contrast enhancement was observed in septa of mass of the other patient with mucinous cystadenocarcinoma, demonstrating that MnDPDP-enhanced MRI gives a better delineation of the tumor, especially in patients with no parenchyma atrophy. It may be difficult to distinguish the lesions simulating the occurrence of malignant neoplasms in patients with focal pancreatitis from malignant tumors. Diminished heteregenous uptake of MnDPDP of the lesions in patients with pancreatitis may be helpful in differential diagnosis.

MnDPDP is used as an organ specific contrast agent orginally designed for liver imaging. Since the agent is taken up by hepatocytes, increased contrast between normal liver parenchyma and metastatic liver lesions can be achieved. MnDPDP facilitates staging of pancreatic tumors by providing easily detection of metastatic lesions to the liver. MnDPDP may improve the staging of pancreatic cancer by increasing the sensitivity of MRI in the detection of liver metastases. MnDPDP-enhanced MRI can improve the accuracy of detection ratio and staging of focal pancreatic lesions, and is a safe and well tolerated noninvasive diagnostic method.

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CLINICAL RESEARCH

Hyperhomocysteinemia and hypercoagulability in primary biliary cirrhosis

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Abstract

AIM: To assess the hypercoagulability in PBC and its relationship with homocysteine (HCY) and various components of the haemostatic system.

METHODS: We investigated 51 PBC patients (43F/8M; mean age: 63±13.9 yr) and 102 healthy subjects (86 women/16 men; 63±13 yr), and evaluated the haemostatic process in whole blood by the Sonoclot analysis and the platelet function by PFA-100 device. We then measured HCY (fasting and after methionine loading), tissue factor (TF), thrombin-antithrombin complexes (TAT), D-dimer (D-D), thrombomodulin (TM), folic acid, vitamin B6 and B12 plasma levels. C677T 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphism was analyzed.

RESULTS: Sonoclot RATE values of patients were significantly ($P < 0.001$) higher than those of controls. Sonoclot time to peak values and PFA-100 closure times were comparable in patients and controls. TAT, TF and HCY levels, both in the fasting and post-methionine loading, were significantly ($P < 0.001$) higher in patients than in controls. Vitamin deficiencies were detected in 45/51 patients (88.2%). The prevalence of the homozygous TT677 MTHFR genotype was significantly higher in patients (31.4%) than in controls (17.5%) ($P < 0.05$). Sonoclot RATE values correlated significantly with HCY levels and TF.

CONCLUSION: In PBC, hyper-HCY is related to hypovitaminosis and genetic predisposing factors. Increased TF and HCY levels and signs of endothelial activation are associated with hypercoagulability and may have an important role in blood clotting activation.

Key words: Homocysteinemia; Hypercoagulability; Primary biliary cirrhosis; Tissue factor; Folic acid

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INTRODUCTION

Primary biliary cirrhosis is deemed to be an autoimmune chronic cholestatic disorder of the liver that primarily affects middle aged women. Currently, the diagnosis of PBC is often made when the patient is still asymptomatic, with abnormal liver biochemistry and/or antimitochondrial antibodies (AMA). The symptomatic patients may have fatigue, generalized pruritus, osteoporosis, fat soluble vitamin deficiencies and portal hypertension[1,2]. The disease generally progresses slowly but survival is less than age- and gender-matched general population and natural history may vary greatly from patient to another[3].

Thrombosis of portal veins has been detected in 40% of PBC livers resected at orthotopic liver transplantation (OLTx) and is correlated with history of bleeding varices[4]. It has been hypothesized that portal veins thrombosis may be responsible for causing development of non-cirrhotic portal hypertension and progression of liver fibrosis[5,6]. A higher incidence of thrombosis of portal venous tree may be promoted by a hypercoagulable state[7] and recent evidence has demonstrated that PBC patients were hypercoagulable on thromboelastography[8,9]. Scarce data are available on the possible causes of this hypercoagulability. In particular, plasma coagulation factors have not been completely evaluated in these patients.

Hyperhomocysteinemia has been found to be associated with a hypercoagulable state and liver fibrosis[10,11]. It has been documented that mean basal and post-methionine load serum HCY levels are significantly higher in patients with PBC than healthy controls[12]. However these findings

need to be confirmed and no data are available on the genetic and acquired causes of such hyperhomocysteinemia and its relationship with hypercoagulability in patients with PBC.

The aim of this study was to investigate basal and post-methionine load serum homocysteine levels, various components of plasmatic coagulation, platelet function and their relationship with hypercoagulability in PBC patients.

MATERIALS AND METHODS

Subjects

Fifty-one consecutive patients with a diagnosis of PBC (43 women and 8 men; age: median 63±13.9 yr (range 20-76) referred to the Gastroenterology Unit of the University of Florence from December 2001 to July 2002, were enrolled. We divided the patients into four staging severity groups: 12 patients in stage I, 11 in stage II, 15 in stage III and 13 in stage IV. Exclusion criteria were renal insufficiency, consumption of alcohol, therapy with steroids, anticoagulants, NSAIDs or methotrexate, folic acid and vitamin B12 supplementation. All patients were stable with no history of bleeding or infectious complications at least 6 wk before evaluation. Routine laboratory investigations included aPTT, PT, fibrinogen, liver function tests (bilirubin, alkaline phosphatase, albumin, aspartate and alanine transaminases), creatinine, cholesterol, iron, calcium, and antimitochondrial antibodies (AMA). All cholestatic patients received vitamin K. All patients had a screening for thyroid-associated disease and intestinal bowel diseases.

One hundred and two healthy subjects with no previous history of liver disease, comparable for age and sex (86 women and 16 men; median 63±13 (range 18-75) yr), recruited from blood donors of our hospital and laboratory volunteers, were used as control group. Patients and controls gave their informed consent to use part of their blood samples for an experimental study.

Experimental procedures

Venous blood samples were collected from the basilical vein after discarding the first 2 mL of blood. We determined HCY plasma levels in the fasting state and 4 h after an oral load with methionine. One hundred mg/Kg of body weight of L-methionine was administered in approximately 200 mL of fruit juice immediately after the fasting phlebotomy.

Sonoclot™ analysis

The haemostatic process in whole blood has been measured by the Sonoclot™ (Sienco Company, Morrison, CO) analysis, a viscoelastic test on whole blood collected in tubes containing 0.129 M sodium citrate. (Saleem 83) Sonoclot analysis was performed within 30 min from the phlebotomy, with 360 μL citrated blood recalcified with 15 μL of calcium chloride (0.25 mol/L). The following variables were analyzed: (1) the clot RATE, i.e. the gradient of the primary slope measured as Clot Signal Units per minute which is an index of clot formation affected by both platelets and coagulation proteins; (2) the time to peak amplitude (TP) (minutes) which reflects the clot retraction away from the surface of the probe and is mainly influenced by the platelet function.

Platelet function analysis

Platelet function was evaluated by the PFA-100 ® device (Dade-Behring) on whole blood collected in tubes containing 0.129 M sodium citrate. The closure times (CT) were determined on duplicate samples (0.8 mL) within 2 hours of collection, using cartridges containing collagen-coated membranes with epinephrine (Col/Epi cartridge) or ADP (Col/ADP cartridge) as previously described[9].

Blood coagulation parameters

D-dimer (D-D), thrombin-antithrombin complex (TAT), tissue factor (TF) and thrombomodulin (TM) plasma levels were evaluated using ELISA method (D-D: Agen, Brisbane, Australia; TAT: Dade Behring, Marburg, Germany; TF: American Diagnostica, Greenwich,CT,USA; TM: Diagnostica Stago, Asnieres, France) on blood samples collected in tubes containing 0.129 M sodium citrate, immediately centrifugated at 4 ℃ and stored in plasma aliquots at -80 ℃ and assayed within two weeks.

Homocysteine assay

To determine HCY, whole blood was collected in tubes containing ethylenediaminotetracetate (EDTA) 0.17 mol/L, immediately put in ice and centrifuged within 30 minutes at 4 ℃ (15000 x g for 15 min). The supernatant was stored in aliquots at -80 ℃ until assay. Homocysteine plasma levels were detected by FPIA method (Abbott, Wiesbaden, Germany).

Vitamin pattern

Serum folic acid and vitamin B12 were measured by radioassay (ICN Pharmaceuticals, NY)). Serum vitamin B6 was measured with HPLC method and fluorescence detecting (Immundiagnostik, Benheim, Germany).

MTHFR polymorphism

Genomic DNA was extracted from peripheral blood leukocytes. C677T polymorphism in the MTHFR gene was carried out on genomic DNA by PCR amplification as already described[13].

Statistical analysis

All analyses were performed with SPSS 10.0 for Windows (SPSS, Chicago, IL, USA). The non-parametric Mann-Whitney test for unpaired data was used for comparisons between single groups. Spearman's rank correlation coefficient (for non-parametric data) was used for the correlation analysis. Fasting and post-methionine hyperhomocysteinemia were defined based on the 95th percentile cut-off of the control population (Fasting: M=15 μmol/L, F=13 μmol/L; Post-methionine: M=38 μmol/L, F=35 μmol/L).

For genetic analysis, Hardy-Weinberg equilibrium was assessed using χ^2 analysis. All probability values are two-tailed with values less than 0.05 considered to be

Table 1 Laboratory characteristics of patients investigated according to the stage of disease[1]

Stage	I	II	III	IV
Patients (*n*°)	12	11	15	13
Alkaline phosphatase (U/L)	273 (50-797)	447 (145-1315)	397 (190-1956)	289 (52-1960)
AST (U/L)	28 (13-6)	38 (25-400)	35 (14-63)	49 (11-144)
ALT (U/L)	37 (14-89)	54 (17-100)	39 (17-72)	47 (8-218)
Total Bilirubin (mg/dL)	0.7 (0.4-2.4)	0.9 (0.2-1.2)	0.9 (0.3-1.5)	1.0 (0.5-1.6)
Cholesterol (mg/dL)	190 (112-235)	220 (104-285)	219 (110-275)	208 (154-314)
aPTT (sec)	32 (27-63)	28 (26-43)	32 (25-37)	32 (26-36)
PT (%)	98 (90-105)	100 (91-120)	99 (81-100)	93 (58-100)
Fibrinogen (mg/dL)	379 (279-650)	380 (268-463)	327 (276-590)	380 (100-586)

[1]Data are expressed as median (range).

Table 2 Platelet and blood coagulation tests

	PBC patients *n* = 51	Controls *n* = 102
Sonoclot RATE (U/min) all pts	29 (14-49)[b]	21 (14-31)
• <150 x 10^3/μL plts (*n* = 22)	30 (14-49)[b]	
• ⩾150 x 10^3/μL plts (*n* = 29)	28 (19-41)[d]	
Sonoclot TP (min) all pts	12 (6-30)[d]	9 (6-15)
• <150 x 10^3/μL plts (*n* = 22)	15 (6-30)[d]	
• ⩾150 x 10^3/μL plts (*n* = 29)	10 (6-24)	
PFA/EPI - CT (secs) [⩾150 x 10^3/μL plts (*n* = 29)]	151.5 (60-222)	146 (111-179)
PFA/ADP- CT (secs) [⩾150 x 10^3/μL plts (*n* = 29)]	106 (61-180)	102 (61-148)
TAT (μg/L)	2.8 (1.5-87.3)[b]	2.5 (1.1-4.2)
D-Dimer (ng/mL)	16.0 (2-173)	23.0 (3-55)
TF (pg/mL)	255.6 (24.8-466.1)[d]	121.9 (24.8-466.1)
TM (μg/mL)	23.7 (2.6-153.5)[d]	13.8 (7.6-23.1)

[b]$P < 0.01$, [d]$P < 0.001$ *vs* Controls; TP = time to peak; PFA/EPI - CT = closure time with epinephrine; PFA/ADP-CT = closure time with ADP. Data are expressed as median (range).

statistically significant.

RESULTS

Laboratory characteristics of patients investigated according to the stage of disease are shown in Table 1.

Blood Coagulation and Platelet Analysis

Sonoclot RATE values of PBC patients were significantly higher than those of controls ($P < 0.001$). In both the 29/51 (57%) PBC patients with a platelet count in normal range and the 22/51 (43%) PBC patients with thrombocytopenia as well as 26/51 (51%) PBC patients, the Sonoclot RATE value was higher than 95% of controls ($P < 0.001$) (Table 2).

Sonoclot TP values were significantly higher only in PBC patients with thrombocytopenia. Indeed, a significant difference of Sonoclot TP ($P < 0.001$) values was observed between PBC patients with a normal platelet count and with thrombocytopenia (Table 2). The hypercoagulable state and the increased TF circulating levels were independent of the disease's stage, and were presented even in the first stage of the disease (data not shown).

PFA-100 closure times after stimulation with epinephrine or ADP were comparable in patients with PBC and controls (Table 2). In 9/51 (17.6%) PBC patients, D-dimer plasma levels were higher than 95% of controls but the difference did not reach the statistical significance ($P = 0.16$).

TAT, thrombomodulin and tissue factor levels were significantly higher in patients than in controls (TAT 2.8±13.9 (1.5-87.3) μg/L *vs* 2.5±0.52 (1.1-4.2) μg/L; TM 23.7±3.5, (2.6-153.5) ng/mL *vs* 13.8±4.4 (7.6-23.1) ng/mL; TF 255.6±223.0 (81.4-1259.6) pg/mL *vs* 121.9±81.9 (24.8-466.1) pg/mL; $P < 0.001$).

Homocysteine

HCY plasma levels, both in the fasting state and post-methionine loading, were significantly higher in patients than in controls (Fasting: 12.1±8.76 (1.5-58.8) μmol/L *vs* 9.9±1.7 (6.4-18.0) μmol/L; Post-methionine 30.1±14.4 (9.2-99.6) μmol/L *vs* 28.0±5.23 (16.4-38.9) μmol/L, $P < 0.001$) (Table 3). Totally, hyperhomocysteinemia (defined as a concentration of fasting and/or post-methionine HCY above 95% of controls) was diagnosed in 23/51 patients (45.1%) (8 only fasting; 4 only PM; 11 both). No significant difference in plasma HCY levels was detected between four staging severity groups.

Folic acid, vitamins B12 and B6

Vitamin deficiencies (defined as a vitamin concentration below the 10th percentile of controls) were detected in 45/51 patients (88.2%).

Deficiency of folate (defined as a concentration less than 6.4 ng/mL) was documented in 39/51 (76.5%); deficiency of vitamin B12 (defined as a concentration less than 243.7 pg/mL) was found in 6/51 patients (11.8%). Deficiency of vitamin B6 (defined as a concentration less than 3.4 pg/mL) was found in 3/51 patients (5.8%). Three patients had low levels of both folic acid and vitamin B12, 1 patient of both vitamin B12 and B6 and 2 patients of both folic acid and vitamin B6.

Vitamins median values are shown in Table 3.

MTHFR C677T polymorphism

The allele frequency of the C677T polymorphism was 0.52 in patients and 0.45 in controls. The distribution of the three genotypes in controls was as follows: TT 17.5 %; CT 55.3%; CC 27.2%. The genotype distribution in patients was as following: TT 31.4%; CT 41.2%; CC 27.4%. The prevalence of the homozygous TT677 genotype was significantly higher in patients (31.4%) than in controls (17.5%) ($P < 0.05$).

Table 3 Homocysteine and vitamin levels in PBC patients and controls

	PBC Patients ($n = 51$)	Controls ($n = 102$)
Fasting HCY (μmol/L)	12.1 (1.8-58.8)[b]	9.9 (6.4-18.0)
Post-methionine HCY (μmol/L)	30.1 (9.2-99.6)[b]	28.0(16.4-38.9)
Folic Acid (ng/mL)	5.3(1.2-13.4)[b]	10.7(5.4-18.5)
Vitamin B12 (pg/mL)	335 (201-977)[a]	304.9(176-427.1)
Vitamin B6 (pg/mL)	6.6 (1-20)[b]	10.0 (3-17)

[a]$P < 0.05$ *vs* controls; [b]$P < 0.001$ *vs* control.

Table 4 TTMTHFR polymorphism and vitamin state in 51 PBC patients *n* (%)

MMTHFR	Low folate, normal HCY	Normal folate, high HCY	Low folate, high HCY	Normal folate, normal HCY	Total *No* patients
CT	8 (38)	0	10 (47.6)	3 (14.45)	21
CC	8 (57)	0	2 (14.5)	4 (28.5)	14
TT	3 (18.7)	2 (12.5)	9 (56)	2 (12.5)	16
Total	19	2	21	9	

Patients with the homozygous TT677 genotype had higher, but not statistically significant HCY levels than those with C677T and CC677 genotypes (TT = 13.3 (1.8-58.8) μmol/L; CT = 12.3 (7.9-42.6) μmol/L; CC = 10.2 (6.0-21.7) μmol/L).

TTMTHFR polymorphism and/or vitamin deficiencies were present in all hyperhomocysteinemic patients (Table 4). In other words, in patients with normal folic acid plasma levels homocysteinemia was normal except for two patients with TTMTHFR polymorphism.

Correlation between the parameters investigated

A significant correlation between Sonoclot rate values, TAT ($r = 0.44$, $P < 0.001$), TF plasma levels ($r = 0.30$, $P < 0.05$) and basal HCY ($r = 0.45$, $P < 0.001$) was observed. Sonoclot rate was significantly higher in patients with high fasting state and/or post-methionine than in the other patients [34 ± 7.4 (22-45) U/min *vs* 26 ± 5.4 (14-49) U/min (Figure 1)]. Moreover, a significant correlation was detected between HCY, TM ($r = 0.54$, $P < 0.001$) and TF ($r = 0.55$, $P < 0.05$).

TAT levels correlated significantly with TF ($r = 0.43$, $P < 0.05$) while HCY plasma levels correlated significantly with cholesterol plasma levels ($r = 0.55$, $P < 0.001$).

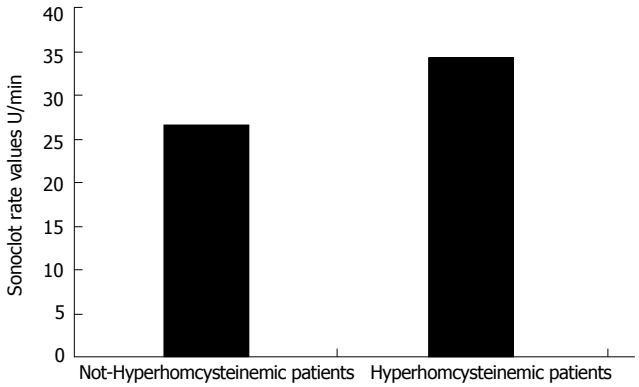

Figure 1 Sonoclot rate values in PBC patients with or without hyperhomocysteinemia.

DISCUSSION

Hypercoagulability in PBC has been previously documented with thromboelastography[8,9], and here we have confirmed this finding with Sonoclot, another technique of analysis of haemostatic process, and demonstrated high TAT circulating levels in 51 PBC patients.

Hypercoagulability may have two different roles in natural history of PBC: it could promote portal veins thrombosis and liver damage and on the other hand it could be responsible for a more favourable prognosis of variceal bleeding[14,15] and a lower blood loss at liver transplantation[11,16-19]. It has been hypothesized that portal veins thrombosis may be responsible for causing development of regenerative nodules and non-cirrhotic portal hypertension and progression of liver fibrosis[5,6]. In PBC patients hypercoagulability may contribute to the high incidence of oesophageal varices in early histological stages associated with the presence of regenerating nodules[20]. A recent study demonstrated that anticoagulant therapy with a thrombin antagonism can reduce fibrogenesis in rat liver[21], it could be interesting to evaluate if a correction of hypercoagulability could reduce the intrahepatic thrombosis formation and development of liver fibrosis and portal hypertension.

Moreover we found that TF might be involved in determination of thrombophilic status, in fact TF levels are higher in PBC patients than in healthy controls and are related to Sonoclot Rate values. TF is a glycoprotein present on the surface of the plasma membranes of monocytes, endothelial cells and smooth muscle cells[22,23], and it is the primary cellular trigger of the coagulation cascade. In our patients clinical conditions that may affect TF concentrations such as infections, neoplasia, or heparin administration have been ruled out. Lymphocyte activation is able to induce tissue factor expression by monocytes[23]. PBC is characterized by an intense biliary and systemic inflammatory CD4+ and CD8+ T cell response[23,25] and may be responsible for tissue factor expression and subsequent elevation of circulating levels.

Our data confirm the finding of elevated homocysteine levels in patients with PBC. Hyperhomocysteinemia has been found to be associated with a hypercoagulable state and liver fibrosis[10]. Here we demonstrated that HCY levels are associated with hypercoagulability and, as reported by others in the setting of thrombotic diseases[26,27], in this study we offer the "*in vivo*" demonstration that in PBC patients high levels of HCY are related to increased TF plasma concentration. TF expression by endothelial cells and monocyte-macrophages may be induced by HCY. This effect has been documented *in vitro* studies, in experimental animals and in hyperhomocysteinemic patients in a specific and dose-dependent manner[28]. HCY may be one of the mechanisms involved in the endothelial stimulation, as documented by the significant correlation with TM levels, which are significantly higher in patients than in controls.

Platelet function has been investigated by two parameters: Sonoclot TP value and PFA-100 closure time. Both of them have been found to be comparable or even higher than healthy controls values. These data are similar to those presented by Pihush *et al*[9].

As regards fibrinolysis, our data demonstrate that the hypercoagulable state in PBC patients is not associated with a comparable activation of fibrinolysis, as no difference in D-D levels was documented between patients and controls. Further studies are necessary to thoroughly investigate fibrinolysis, and in particular its main inhibitor, plasminogen activator inhibitor-1 (PAI-1), which may be released by stimulated monocytes and endothelial cells[29]. This behaviour of fibrinolytic system may contribute to determine a prothrombotic state.

In this study the main possible genetic and acquired alterations of HCY metabolism in PBC were investigated. We demonstrated the presence of vitamin deficiencies related to methionine metabolism (folic acid, vitamin B6 and vitamin B12) in about 90% of patients. In particular, the majority of patients had a folate deficiency. This is a novel finding that arises from the question about the potential clinical utility of a low-cost vitamin supplementation in these patients. It should be investigated if folate supplementation could correct hyperhomocysteinemia and/or hypercoagulability. Folate levels may be low due to inadequate dietary intake, malabsorption, increased utilization or to effects of drugs[30]; in our patients we excluded the presence of drugs able to interfere with folate absorption, such as methotrexate. Disease activity may contribute to increased demand for folate due to inflammation[32]. However, we found no correlation between disease activity, as measured by histological stage, and folate or HCY levels. Therefore, these data may point to inadequate intake as a significant factor that affects folate levels. Another hypothesis is the presence of an impairment of the folate enterohepatic circulation. This cycle plays an important role in the homeostasis of folic acid. It has been demonstrated that the interruption of bile circulation by bile drainage leads, to a rapid fall in serum folate levels to 30% of base line within 4-6 h[33]. Cholestasis could make this system of vitamin reutilization ineffective, and could induce folate low serum levels.

No data are available on the prevalence of C677T 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphism in PBC patients. This polymorphism is one of the most frequent genetic factors responsible for the alteration of HCY levels, especially in the presence of a suboptimal folate status[34,35]. We found a significant higher prevalence of the homozygous TT677 genotype in PBC patients. In these patients MTHFR has a reduced activity caused by a C-T substitution at nucleotide 677[36]. However, in order to investigate the prevalence of this genetic polymorphism in PBC further larger studies are needed.

In PBC serum cholesterol levels markedly increase with worsening of cholestasis[37,38]. In this study, as in others,[39,40] a significant in vivo association between HCY and cholesterol circulating levels was found, suggesting another possible explanation, together with cholestasis, for hypercholesterolemia in this disease.

Recently, in cultured human hepatocytes it was demonstrated that hyperhomocysteinemia determines an oxidative stress of the endoplasmic reticulum which activates the sterol regulatory element-binding proteins (SREBPs)[41]. The activation of SREBPs is associated with increased expression of genes responsible for cholesterol/triglyceride biosynthesis, and may be an explanation to our findings.

In conclusion, hypercoagulability and hyperhomocysteinemia exist in patients with PBC, and there is an association between these two parameters. TF may have a role in determination of blood clotting activation and hyperhomocysteinemia is related to hypovitaminosis and genetic predisposing factors. Further studies are needed to clarify if hyperhomocysteinemia and hypercoagulability may have a role in progression of liver damage and if they may be influenced by vitamin supplementation.

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RAPID COMMUNICATION

Soluble CD40 ligand in prediction of acute severe pancreatitis

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Abstract

AIM: To assess the early predictability of the soluble CD40L (sCD40L) in pancreatitis severity.

METHODS: Between February 2000 and February 2003, 279 consecutive patients with acute pancreatitis were prospectively enrolled in our study. In this report, 40 patients with mild and 40 patients with severe pancreatitis were randomly studied. sCD40L concentrations were measured 48 hours after admission.

RESULTS: sCD40L levels were significantly higher 48 hours after admission in severe pancreatitis than in mild pancreatitis. Using a cutoff of 1000 pg/L, the sensitivity and specificity of sCD40L to detect a severe course of the disease were 78% and 62% respectively compared to 72% and 81% for CRP. Logistic regression analysis found that CRP was the only statistically significant marker able to detect a severe course of the disease.

CONCLUSION: These findings indicate that CRP remains a valuable marker to determine the severity and prognosis of acute pancreatitis whereas sCD40L levels should be assessed in further studies.

Key words: Acute pancreatitis; Prognostic factor; sCD40

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INTRODUCTION

Acute pancreatitis (AP) is a disease of variable severity. While approximately 80% of patients experience mild attacks, the remaining 20% still suffer from severe disease, with a mortality rate as high as 30%[1,2]. Since treatment of patients with severe disease is currently mostly supportive and directs at either prevention or control of the local and systemic complications, the early prediction of the severity of AP is the main goal of clinicians in charge of such patients[2]. Multifactorial scales including Ranson and APACHE II scoring systems have been used in acute pancreatitis since the 1980s. Ranson scale has been used in the 1980s in virtually all studies dealing with acute pancreatitis[3,4]. The list of 11 numerical parameters proposed by Ranson has no been fully discussed because only 5 criteria are obtained at admission and the remaining 6 during the subsequent 48 hour period, delay the performance of an early prediction. Moreover, the complexity of these scales and perhaps the fact that CT scanners are not available in every hospital account for the increasing interest in serum markers[5].

The pathophysiology of acute pancreatitis includes the activation and release of pancreatic enzymes in the interstitium, pancreatic autodigestion, and a multiple organ dysfunction following the release of these enzymes into the systemic circulation[6,7]. There is evidence that synthesis and release of pro-inflammatory cytokines and chemokines are also responsible for local and distant injuries[8,9]. If the concentration of these biological factors is correlated to the severity of the disease and if they are detected before the occurrence of multiple organ dysfunction, these factors might be important for the rapid scoring of the disease severity in the acute phase[5].

CD40, a proteic member of the tumour necrosis factor receptor family, is expressed on the membrane of a variety of cells, including B lymphocytes, monocytes, biliary and acinar cells as recently shown by our group[7,10]. CD40 binds to its ligand CD40L, a membrane glycoprotein, to mediate major immunoregulatory signals involved in auto-immune disease, graft rejection, inflammatory bowel disease, development of atherosclerosis and acute experimental pancreatitis[11]. We postulated that CD40-CD40L interactions might also play a role in the pathogenesis of human pancreatitis and that the soluble form of CD40L

Table 1 Characteristics of the population

	Control group	Mild pancreatitis	Severe pancreatitis	*P* value
n =	10	40	40	
Male/female	5/5	27/13	31/9	0.33
Age (year)	33.5 (31.2-35.3)	52.5 (51.5-62.7)	58.5 (56.1-66.6)	0.21
Serum amylase (U/L)	98 (75-128)	356 (352-892)	474 (451-1238)	0.34
Delay after onset (hours)	-	20.1 (18.2-22.4)	22.2 (19.5-23.2)	0.58
Cause of AP				
-biliary	-	17	21	0.5
-alcohol	-	14	16	0.6
-other	-	2	1	0.6
-idiopathic	-	7	2	0.15
Ranson > 3	-	8	19	0.017
Ranson value	-	2 (1.5-2.1)	2 (1.9-2.6)	
sCD40L (pg/L)	22 (16.3-28.1)	795 (692-1301)	1215 (1186-1753)	
CRP levels (mg/L)	12.2 (9.3-15.5)	42.5 (50.1-101.7)	225.5 (199.8-319.4)	0.0007
CT scan				
-normal	-	5	0	0.021
-edematous	-	28	19	0.04
-necrosis	-	19	9	0.02
-collection	-	8	27	0.0001
Death	-	0	3	0.07

Values are expressed as median values (CI 95%).

(sCD40L) levels might be correlated to the severity of the disease. The purpose of this study was to assess the early predictability of sCD40L in pancreatitis severity.

MATERIALS AND METHODS

The study protocol was approved by the local Ethics Committee. Upon admission, all patients with acute pancreatitis meeting the criteria of inclusion were identified and proposed to be included in the study. Before inclusion, each patient gave an informed consent. Between February 2000 and February 2003, 279 consecutive patients with acute pancreatitis were prospectively enrolled in our study. We assessed the role of sCD40L in the prediction of the severity of pancreatitis in this preliminary study. Consequently, 40 patients with mild and 40 patients with severe pancreatitis were randomly evaluated in the current study (Table 1). Ten healthy controls from our own divisions were also used for comparison. Inclusion criteria were typical abdominal pain associated with serum amylase twice the upper limit value (range: 26-128 U/L). The diagnosis was subsequently confirmed by CT scan with a Picker 1200 or a PQ-2000 instrument. Before scanning 150 ml of diluted oral contrast material (Telebrix Gastro, France) was administered to visualize the gastrointestinal tract. Intravenous contrast material (Omnipaque 350, Shering, Germany) was injected and multiple scans were obtained from the pancreas to the pelvis as previously described[12]. Pancreatic necrosis was considered significant if there was > 30% necrosis of the gland. Pancreatitis severity was defined according to the Atlanta classification and included organ failure (e.g.,shock, pulmonary insufficiency, renal failure), the presence of local complications (pancreatic necrosis, pseudocysts, abscess), or both[13]. Blood samples drawn 48 hours after admission were used to measure CRP and sCD40L using commercially available kits (Behring, Marburg, Germany and Bender MedSystems Vienna, Austria).

Statistical analysis

Serum markers were compared during severe and mild attacks by univariate analysis (Student' *t*-test, Mann-Whitney). SCD40L, CRP and Ranson scores were evaluated by receiver operating characteristics (ROC) curves for selected sCD40L and historically determined (CRP and Ranson) cutoff levels[3,4,5]. The cutoff for CRP (150 mg/L) and Ranson (severe > 3) was chosen according to the literature[4,5]. The cutoff for sCD40L was chosen according to its ROC curve. A logistic regression analysis for the binary outcome of mild or severe pancreatitis was performed. $P < 0.05$ was considered statistically significant. Statistics calculations were done using SPSS Software package (Chicago, USA).

RESULTS

The delay between the onset of abdominal pain and admission ranged from 7 hours to 2 d (Table 1). The aetiology of acute pancreatitis was biliary in 38 patients (48%), alcoholism in 30 (38%), drug-induced pancreatitis in 2 (3 %), hypertriglyceridemia in 1 (2 %) and idiopathic in the remaining 9 patients (11%) (Table1).

CT scan was obtained within 36 hours after admission in each patient and showed a normal pancreas in 5 patients, edema in 47 patients and necrosis in 28 patients (Table 1). Acute fluid collections were found in 35 patients (43 %) (Table1). The Ranson score established at 48 hours was $\geqslant 3$ in 27 patients (33 %), 19 of whom had severe pancreatitis and 8 mild pancreatitis. Sensitivity, specificity, positive predictive value, and negative predictive value of Ranson

Table 2 Performances of sCD40L, CRP and RANSON score

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
sCD40L (cutoff 1000 pg/L)	77	62	63	74
CRP (150 mg/L)	72	81	80	74
RANSON Score (>3)	48	80	70	60

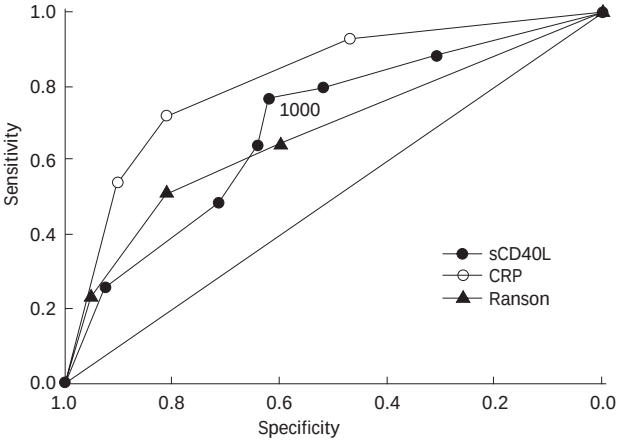

Figure 2 sCD40L (400, 800, 1000, 1200 1400 and 2000 pg/L), CRP (50, 150 and 200 mg/L) and Ranson (Ranson score of 2, 3 and 4) ROC curves with selected or historically determined cutoffs in patients with mild and severe pancreatitis.

score to predict a severe attack were 48% and 80%, and 70% and 60% respectively (Table 2). Three patients group with severe pancreatitis (7.5 %) died, two of whom were of biliary origin (Table 1).

Healthy controls had very low levels of sCD40L as well as CRP (Table 1). By comparison, levels of sCD40L and CRP were 795 pg/L (CI 95 %: 692-1301) and 42.5 mg/L (CI95%: 50-101) in patients with mild pancreatitis and 1215 pg/L (C I95% 1186-1753) and 225 mg/L (CI 95 % 199-319) ($P<0.0007$ and $P<0.0001$) in patients with severe pancreatitis respectively (Table 1 and Figure 1). No significant correlation was found between serum levels of sCD40L and the nature of the complications that occurred in patients with severe pancreatitis. There was only a trend towards correlation between sCD40L and the occurrence of multiple organ failure. The three patients who died from severe pancreatitis had intermediate levels of sCD40L (range from 184 to 236 pg/L). The sensitivity, specificity, positive predictive value, and negative predictive value of CRP to predict a severe attack were 72% and 81% respectively and 80% and 74% respectively (Table 2). Ranson score, CRP and sCD40L results were evaluated by their ROC curves for selected cutoff levels (Figure 1). Using a cutoff of 1000 pg/L for sCD40L as defined by the ROC curve, the sensitivity, specificity, positive predictive value and negative predictive value of sCD40L to predict a severe attack were 77% and 62% respectively, whereas the positive and negative predictive values were 63 % and 74% (Table 2). Logistic regression analysis for the binary outcome of mild or severe pancreatitis was performed to better delineate the performance of Ranson score, CRP and sCD40L (Figure 2). The forward stepwise analysis

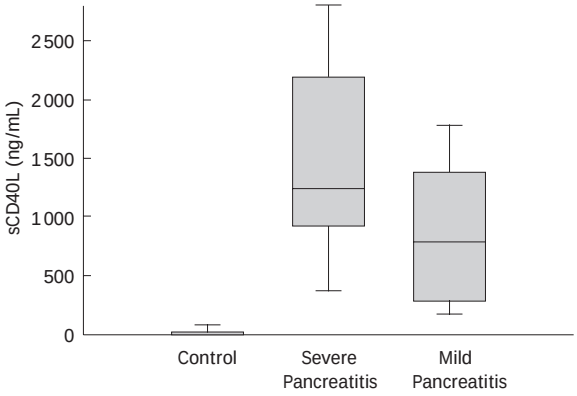

Figure 1 Soluble CD40L concentrations in healthy controls, severe and mild pancreatitis patients. Horizontal bars delineate the mean value.

found that CRP at d 2 was the only statistically significant parameter to predict a severe disease ($P<0.001$).

DISCUSSION

Predicting the course of acute pancreatitis remains a challenge for the physician[2]. Moreover, the 48-hour delay necessary to collect the standard scores and the complexity of multifactorial scales have prompted many physicians to investigate new markers[5]. CRP is an acute phase protein produced by the liver during inflammatory conditions that was first described in 1930[3]. In the mid-1980s, several studies showed that CRP could be considered as a valuable prognostic factor for severe pancreatitis[3,5,14]. Indeed, a better understanding of the pathophysiology of acute pancreatitis may contribute to a better selection of a specific prognostic marker. To this end, CD40-CD40L interaction, a major pro-inflammatory system widely distributed on a variety of leukocytes, has been recently studied in experimental rodent pancreatitis[7]. The expression of CD40 and its ligand were detected on pancreatic acinar cell surface, and peripheral T lymphocytes showed a progressive increase in the expression of CD40L during the course of experimental pancreatitis. Taken together, we thought that these findings might be applied to human pancreatitis. In the current report where 40 patients with mild and 40 patients with severe pancreatitis were randomly evaluated, we showed for the first time that the levels of the soluble form of CD40L (sCD40L) were higher in severe than in mild pancreatitis 48 hours after admission. The usefulness of this potential new marker has been compared to CRP, the most widely accepted serum marker that is used on a daily routine in almost all hospitals[3,7] and to Ranson score. We found that the sensitivity of sCD40L to predict a severe course was better than that by CRP. The negative predictive value of sCD40L that allows the physician to really exclude a severe disease was superior than that by CRP. A marker characterized by a better negative predictive allows cost savings since it can avoid useless observation time in emergency room or intensive care unit[15,16]. However, logistic regression analysis found that CRP was the only statistically significant marker able to identify a patient who developed a severe course of the disease. One explanation for this result is that our study was limited by the

small number of patients evaluated. Consequently, correlation between the levels of sCD40L and the occurrence of complications or death for example could not be studied.

Of note, Ranson score appeared insufficiently predictive of severe attacks, a feature already reported in several previous prospective studies[14,17,18]. To predict the severity of pancreatic disease itself, before the occurrence of multiple organ dysfunction, numerous other factors besides CRP have been evaluated so far with various performances. These factors are either enzymes released from the pancreas such as trypsinogen activation peptide[16] or mediators induced by inflammation in the pancreas itself or in remote organs such as procalcitonin[19], adhesion molecules[20] or cytokines[21]. For most of them, the higher the blood concentrations of these enzymes or mediators are, the more severe the disease is. Soluble CD40L could be one of the next markers of pancreatitis severity but further larger studies are needed to validate its clinical use in the early assessment of pancreatitis severity.

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RAPID COMMUNICATION

Association of the HLA-DRB1*0701 allele with perinuclear anti-neutrophil cytoplasmatic antibodies in Mexican patients with severe ulcerative colitis

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Abstract

AIM: To determine the association between the HLA-DRB1 alleles and perinuclear anti-neutrophil cytoplasmatic antibodies (p-ANCA) positive in Mexican patients with ulcerative colitis (UC).

METHODS: Ninety Mexican mestizo patients (45 females) with UC, confirmed by biopsy, were studied. High resolution HLA typing was performed by PCR-SSO reverse dot blot and PCR-SSP. Molecular typing techniques were applied to define HLA-DRB1 alleles. Enzyme-linked immunosorbent assay and immunofluorescence techniques were used to detect p-ANCA.

RESULTS: Forty-eight (53%) UC patients were positive for p-ANCA by ELISA and IF. We found that p-ANCA-positive UC patients had a significantly increased frequency of HLA-DR7 compared with p-ANCA-negative controls (22% *vs* 5.1%; pC = 0.02, OR = 5.2, CI 95%: 1.06-37.82). Disease activity was scored as severe in 20 patients, moderate in 8, mild in 14 and no activity in the remaining 38 patients according to the Truelove and Witts criteria. Subgroup analysis showed a significantly increased frequency of the HLA-DRB1*07 allele in 15 of 20 UC patients with severe activity of UC and p-ANCA positivity [100% *vs* 0%; pC = 0.0000001; OR = 35]. No significant differences were found between p-ANCA positive patients, HLA-DR alleles and other clinical features such as extraintestinal manifestations, proctocolectomy and extension.

CONCLUSION: The HLA-DRB1*07 is associated with p-ANCA positive UC Mexican patients.

Key words: HLA-DR; p-ANCA; Ulcerative colitis; Mexicans

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INTRODUCTION

Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) have been found consistently in patients with ulcerative colitis (UC)[1,2] however, their pathogenic role is still uncertain. The prevalence of p-ANCA in UC varies greatly from 39 to 80 percent in the ethnic group studied[2].

A genetic involvement in UC is supported by observations on familial aggregation of the disorder and concordance in monozygotic twins[3]. Considering the central role of the immune system in mediating the tissue damage in IBD, genes that participate in the development and regulation of the immune response, such as the HLA class II genes, are candidates for conferring the genetic susceptibility. Putative associations of UC with the polymorphic genes of the major histocompatibility complex (MHC) located on the short arm of the chromosome 6 suggest a role of these in the genetic susceptibility to develop UC in several populations of different ethnic and geographic background[4].

UC is a clinical and genetic heterogeneous disorder described in several ethnic groups[5-7]. An association between HLA-DR alleles and UC, in particular p-ANCA positive has been reported in patients from Italy[6], China[8] and United States[9].

Mexican mestizo individuals have a proportion of 56% native American Indian genes, 40% Caucasian genes, and 4% African genes[10], thus they are suitable to study the role of ethnicity in the susceptibility to develop UC and

p-ANCA positive in UC. Mestizo represents a complex mixture of European (Caucasian) and American native inhabitants (mongoloid) genetics, and constitutes the core of the Mexican and the Latin American populations.

The purpose of this study was to determine the association between the HLA-DRB1 alleles and p-ANCA positive in Mexican mestizo patients with UC.

MATERIALS AND METHODS

Patients

Ninety Mexican Mestizo patients with ulcerative colitis confirmed by histology were studied. Details of demographic and clinical characteristics of UC were obtained by a questionnaire, review of records and personal interview. No patient had a family history for UC. Disease extension was defined by colonoscopy. Thus, disease was classified either as extensive colitis (inflammation proximal to the splenic flexure) or distal colitis. Disease activity was scored by Truelove and Witts criteria[11].

Blood samples were obtained from 99 Mexican mestizo healthy, unrelated individuals with no family history of UC, ethnically matched to patients with UC as controls. Mexican mestizo individuals included in the present study accounted for 56% native American Indian genes, 40% white genes and 4% Black genes[11].

Methods

HLA typing Genomic DNA was isolated from peripheral venous blood by a modified "salting out" technique[12], precipitated with ethanol and resuspended in sterile distilled water at a final concentration 0.1-1.0 μg/μL before use.

Generic HLA-DRB1 typing was performed by PCR-SSO reverse dot blot hybridization (Amplicor, Hoffmann La Roche, Basel, Switzerland). High resolution HLA typing was performed by dot blot hybridization of amplified DNA with sequence-specific oligonucleotide probes labeled with digoxigenin di-deoxy-uridine-triphosphate (Dig-11-ddUTP). Information about DRB1 sequence was obtained from the 12th International Histocompatibility Workshop[13].

Indirect Immunofluorescence (IFL) Detection of ANCA was done according to the recommendations of the international workshop[14]. Human neutrophils were isolated from peripheral blood from patients and normal healthy volunteers by gradient centrifugation in 2% methylcellulose solution. The slides were fixed in 98% ethanol at 4℃ for 5 min and dried quickly in air. After wetting with phosphate-buffered saline (PBS) in a dilution of 1:40 and diluted two fold until dilution reached 1:320. Following incubation for 1 h at room temperature, the slides were washed three times with PBS and bound antibodies were incubated and detected with fluorescein isothiocyanate (FITC)-conjugated F(ab') rabbit anti-human IgG, diluted 1:100 in PBS for 30 min at room temperature. Subsequently, the slides were washed three times with PBS and covered with glycerin-phosphate-buffered saline. A titer $\geqslant$1:80 was considered positive, based on the results obtained from healthy controls.

Antigen-specific enzyme-linked immunosorbent assays (ELISA). This method was performed to identify the putative antigens recognized by the p-ANCA positive sera. Human Lf, MPO or cathepsin G was diluted to 2 μg/mL in carbonate buffer (pH 9.6) and 100 μL of each solution was placed in the wells of a 96-well microplate and left for 24 h at 4℃. After the plate was washed three times with PBS, 100 μL of diluted sera (1:100 in PBS containing 0.1 % Tween 20 and 0.1 % skim milk) was added to each well, and the plate was incubated for 1 h at 37℃. After washing, the enzyme reaction was performed, and color development was measured with a microplate colorimeter. Optical density values >2 SD were considered positive[15].

Statistical analysis Gene frequencies were compared using a 2×2 contingency table and Chi square test. Odd ratios have been calculated for the disease in carriers of specific alleles. Comparisons of allele frequencies between subgroups were performed using the EPI-INFO statistical package (Version 5.0; USD incorporated 1990, Stone Mountain, Georgia). All p values quoted were corrected by the Bonferroni test for multiple comparisons, while the number of alleles studied was taken into account. Statistical significance was considered when $P<0.05$.

RESULTS

Clinical features

The average disease duration was 7.2±6.6 years. Extensive colitis was present in 60% and distal colitis in 40%. Disease activity was scored as severe in 20 patients (25%), moderate in 8 (10%), mild in 14 (18%) and no activity in the remaining 38 patients (47%). Extraintestinal manifestations were present in 40%: arthritis (14.6%), primary sclerosing cholangitis (10.4%), sachroiliitis (6%), erythema nodosum (6%), ankylosing spondylitis (1.5%) and aphthous ulceration (1.5%). Fourteen patients (15.5%) required colectomy due to refractory medical therapy.

Positive p-ANCA

Positive p-ANCA was detected by IFL and ELISA methods in 48 of 90 patients with UC (53%) whereas c-ANCA was detected in 4 patients with UC. The frequencies of positive p-ANCA were increased significantly in the patients with UC as compared to controls. Titers of p-ANCA ranged 1:80-1:320.

HLA distribution

Patients with positive p-ANCA showed a significantly increased frequency of the HLA-DRB1*07 as compared to p-ANCA negative patients (pC = 0.02, OR = 5.2, CI 95 %: 1.06-37.82) and healthy controls (pC = 0.04, OR = 2.9, CI 95 %: 1.01-20.56). The remaining HLA-DR alleles did not show statistically differences between UC patients with positive p-ANCA and negative p-ANCA as shown in Table 1.

Subgroup analysis showed a significantly increased frequency of the HLA-DRB1*07 allele in patients with severe activity of UC and positive p-ANCA (15/15) as compared to 5 patients with severe disease and negative p-ANCA and no HLA-DRB1*07 [100 % *vs* 0%; pC = 0.0000001;

Table 1 Gene frequencies (g.f.) of HLA-DR alleles in patients with UC and healthy controls

HLA	p-ANCA + N = 96		p-ANCA – N = 84		Healthy controls N = 198	
	n	g.f.	*n*	g.f.	*n*	g.f.
DR7	21	0.218[1,2]	4	0.047	22	0.111
DR2	16	0.166	13	0.154	14	0.090
DR4	14	0.145	17	0.202	47	0.237
DR1	13	0.135	12	0.142	10	0.050
DR8	10	0.104	11	0.130	33	0.165
DR5	8	0.083	11	0.130	20	0.100
DR6	6	0.062	9	0.107	21	0.109
DR3	5	0.052	5	0.059	11	0.055
DR10	3	0.031	2	0.023	1	0.005

N = Number of chromosomes *n*= number of alleles.
[1]p-ANCA positive *vs* negative: pC = 0.02, OR = 5.2, CI 95%: 1.06-37.82.
[2]p-ANCA positive *vs* controls: pC = 0.04, OR = 2.9, CI 95%: 1.01-20.56.

OR=35]. No significant differences were found between p-ANCA positive patients, HLA-DR alleles and other clinical features such as extraintestinal manifestations, proctocolectomy and extension.

Disease activity

Data about disease activity were collected without knowledge of the results of ANCA testing. Interestingly, a significant association was found between severe activity of the disease and positive p-ANCA [75 % *vs* 25 %; pC = 0.00008; OR = 11; CI 95 %: 4.0-34.0]. No association was found between moderate, mild or inactive disease and the presence of p-ANCA (Table 2).

Extension We found that 22 UC patients with extensive colitis (47.8 %) were positive for p-ANCA while 19 patients with distal colitis were positive for p-ANCA (55.8 %). No statistical association was found between p-ANCA positivity and the extension of the disease [*P* = 0.47; OR = 0.72; CI 95 %: 0.27-1.94].

Proctocolectomy Seven patients with proctocolectomy were positive for p-ANCA as compared to 34 patients (51.5 %) who did not undergo the surgical procedure and were p-ANCA positive. No association was found between both groups [*P* = 0.91; OR = 0.94; CI 95 %: 0.26-3.43].

EIMs Futhermore, association was no found between each of the extraintestinal manifestations such as arthritis, primary sclerosing cholangitis, sachroiliitis, erythema nodosum, ankylosing spondilytis or aphthous ulceration and the presence of p-ANCA.

Type of treatment We followed up 80 patients treated with corticosteroids (local and systemic), 5-ASA (local and systemic) and azatioprin, and 10 untreated patients who served as controls. No association was found between p-ANCA positivity and any kind of medical treatment.

The prevalence of p-ANCA was higher in treated patients (45 %) than in untreated patients (6 %), probably reflecting the greater severity of the cases that required surgical treatment. After stratifying patients according to p-ANCA status, prevalence rates were not different as far as age, gender, age at first diagnosis, disease extension, surgery, extraintestinal manifestations were concerned.

No association was found between the number of clinical relapses and p-ANCA positive determination (data not shown).

Table 2 Clinical features of UC patients with positive and negative p-ANCA

Clinical feature	p-ANCA + *n* = 48	p-ANCA - *n* = 42	*P* value
Age at diagnosis (yr)	31 ± 8	33 ± 7	0.34
Gender (F/M)	23/25	22/20	0.62
Disease duration (yr)	7.4 ± 6.3	6.8 ± 6.2	0.45
Clinical relapses	2 ± 1	3 ± 2	0.25
Medical Treatment			
5-ASA	90%	85%	0.75
Corticosteroids	44%	45%	0.90
Azatioprine or 6-MP	24%	22%	0.82
Extensive colitis	29	27	0.47
Distal colitis	19	15	
Colectomy	7	7	0.94
No Colectomy	41	35	
EIMs present	21	25	0.67
EIMs absent	27	17	
Disease activity			
Absent	19	21	0.82
Mild	9	10	0.71
Moderate	5	6	0.65
Severe	15	5	0.000008

[a]OR = 11; CI 95%:4.0-34.0.
n = Number of patients.

DISCUSSION

This study provides evidence of the important role of MHC genes class II in the development of autoantibodies in patients with ulcerative colitis. The relevant finding was the increased frequency of the HLA-DRB1*07 allele in UC patients with positive p-ANCA compared with UC patients with negative p-ANCA. This association has not been reported in other ethnic groups. Several studies on Caucasians have demonstrated the association between the HLA-DR2 and the presence of p-ANCA positive UC patients from United States[16], Germany[17] and Italy[18]. In Chinese patients, a strong association is found between the HLA-DQ alpha 1c allele and the presence of ANCA positive[8].

On the other hand, other studies have not found association of the presence of positive p-ANCA and HLA-DR alleles in Jewish[2], French[18] and Korean[19] UC patients. Previous Mexican studies have reported association between HLA-DR1 and polymorphisms in the promoter region of tumor necrosis factor alpha and the genetic susceptibility to develop UC in this population[7,20].

The HLA-DRB1*07 has not been reported to have an association with positive p-ANCA in patients with UC from other ethnic groups. Interestingly, this allele is associated with chlorpromazine-induced lupus anticoagulant in patients with chronic psychiatric disorders[21]. We also found that the HLA-DR7 was associated with the production of anti-phospholipid antibodies in a group of Mexican mestizo patients with systemic lupus erythematosus[22]. The differences in the associated alleles could also be explained as a result in the genetic variation due to the role

of ethnicity in certain groups.

Finally, the association of the HLA-DRB1*07 allele with p-ANCA may be a result of immunoregulatory mechanisms related to the antigen presentation of protein fragments (autoantigens) to T cells, and may also play an important role in the development of autoimmunity, including the production of several autoantibodies such as lupus anticoagulant and anti-phospholipid antibodies as mentioned above.

This novel association between HLA-DRB1*07 allele and positive p-ANCA in Mexican UC patients suggests that this allele could be a marker of severe activity in patients with UC and plays a role as genetic marker for the production of different kind of autoantibodies in our population.

In conclusion, the HLA-DRB1*07 allele plays a genetic role in the production of p-ANCA in Mexican mestizo patients with ulcerative colitis.

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RAPID COMMUNICATION

Elevated plasma cryofibrinogen in patients with active inflammatory bowel disease is morbigenous

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Abstract

AIM: To investigate the role of cryofibrinogen (CF) in active inflammatory bowel disease (IBD).

METHODS: CF was assayed in 284 subjects: 61 with active and 63 with inactive ulcerative colitis (UC), 45 who had proctocolectomy, 35 with active and 20 with inactive Crohn's disease (CD), 40 with other diseases and 20 healthy controls. Trypsin inhibitor (TI) and TI antibody (TI-Ab) were measured in plasma and CF complex by ELISA.

RESULTS: CF in active UC was strikingly high compared with all other groups (χ^2<0.001). Similarly, CF was significantly higher in active CD than in inactive CD or in controls (χ^2<0.01). In UC, high CF and TI-Ab were associated with the need for operations. Further, high CF, CF/fibrinogen ratio, low TI and high TI-Ab in plasma were associated with disease activity or refractoriness to medication. Elevated CF was not associated with acute reactants like C-reactive protein and white blood cell counts except for erythrocyte sedimentation rate, suggesting that elevated CF was not a consequence of acute inflammation.

CONCLUSION: Elevated CF in active IBD appears to be morbigenous. CF promotes IBD via two main mechanisms, quenching of TI (an anti-inflammatory substance) and impairing microvascular perfusion by forming protein aggregates. CF may also serve as a biomarker of chronic IBD. Additional studies are warranted to fully evaluate the role of CF in IBD and the outcome should contribute to a better understanding of the pathogenesis of IBD.

Key words: Inflammatory bowel disease; Cryofibrinogen; Trypsin inhibitor; Trypsin inhibitor antibody; Acute colitis

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INTRODUCTION

In 1955, Korst and Kratochivil[1] for the first time described "cryofibrinogen (CF)" as an abnormal protein, which is reversibly cold-precipitable in anticoagulated blood and could form fibrin and clot with thrombin. Now, CF is known as a cold-precipitable protein complex composed of fibrin, fibrinogen and fibrin split products found in plasma but not in serum of some individuals[2-4]. Further, cryofibrinogenaemia is now suspected to be associated with acute and chronic inflammatory diseases, lymphoproliferative disorders, and vascular complications[2-8]. However, it is true to say that up to now, CF has been best known as a risk factor for thrombo-embolic complications[2-9].

Regarding inflammatory bowel disease (IBD) and CF, several independent risk factors for thrombotic vascular diseases have recently been reported in IBD[9-13]. For example, it is known[11] that granulomatous vasculitis and fibrin deposition are components of the inflammatory process in Crohn's disease (CD). Similarly, presence of micro-thrombi in rectal biopsy specimens from patients with either ulcerative colitis (UC) or CD has been reported[12]; the incidence of systemic thrombo-embolic events in patients with IBD is known to be higher than that in the general population[13], reflecting a primary coagulation abnormality in patients with IBD[9-13].

Another biochemical substance, which was investigated in this study in relation to IBD, is trypsin inhibitor (TI). TI is best known for inhibiting trypsin to spare bystander tissues from unwanted damage[14]. Additionally, the literature provides convincing evidence for an anti-inflammatory action by TI as one of the body's naturally occurring immunoregulators[14-16] that include suppression of TNF generation[7]. In view of the above background, in

this study we hoped that finding the relationships between IBD, CF and TI might contribute to the understanding of the pathogenesis of IBD.

MATERIALS AND METHODS

Subjects

Between 1994 and 2001, a total of 284 subjects were investigated in this study. The demography of the study populations is presented in Table 1. There were 169 patients with UC (61 with active UC, 63 with remitted UC and 45 had proctocolectomy), 55 with CD (35 with active CD and 20 with remitted CD), 40 disease controls and 20 healthy controls.

Determination of disease severity and ongoing medications

Patients with active UC had bloody diarrhoea more than 6 times/d, a score of at least 14 on Lichtiger's clinical activity index (CAI)[17] and colonoscopy revealed Matts' s endoscopic grade 3[18]. Patients with active UC received total parenteral nutrition (all had severe UC) together with 5-aminosalicylic acid (5-ASA) or sulphasalazine orally and intravenous (iv) prednisolone (30-80mg/d) except for 5 patients who had their first UC episode before starting drug therapy. The remaining 45 patients with UC who had proctocolectomy with ileal pouch anal anastomosis (IPAA) at least 6 mo prior to this investigation represented the post-operative UC group. None of the 55 patients with CD received steroid therapy. Patients with active CD ($n=35$) had a mean Crohn's disease activity index (CDAI) of 266[19]. The subgroup with inactive CD ($n=20$) had a CDAI of less than 150 (clinical remission level). Forty age and sex matched hospital patients with other diseases were included as a disease control group (Table 1). Of these, 10 had non-IBD acute colitis caused by bacteria [enteropathogenic *Escherichia coli*, Vero toxin (-) in 6, *Salmonella enteritidis* in 3, and *Klebsiella peumoniae* in one], 4 with rheumatoid arthritis, 4 with primary biliary cirrhosis, 2 with autoimmune hepatitis, 2 with Bechet's disease, 2 with systemic lupus erythematosus, and 1 with polymyositis in autoimmune and collagen diseases, 4 with colonic polyps, 3 with chronic hepatitis C, 3 with hypercholesterolemia, 3 with peptic ulcers who were also *Helicobacter pylori* positive, and 2 with gastric cancer in non-acute and non-autoimmune state. Twenty healthy subjects who were in the same age range as patients were included as a healthy control group.

Measurement of fibrinogen and cryofibrinogen

Blood samples were obtained in the early morning fasting state. A 10 mL of whole blood was collected for cryoprecipitate measurements. The blood sample was pre-warmed at 37 ℃ in a plastic syringe and then was transferred into 2 plain glass tubes at 37 ℃; one tube contained 3.8% sodium citrate solution (9:1; vol/vol blood/citrate ratio), the other tube contained no anti-coagulant.[20] The tubes were incubated at 37 ℃ for 1 h and then were centrifuged at 1500g for 30 min to separate plasma or serum from cells.[21] Fibrinogen was measured by immunoturbidimetric assay according to a published method.[22] Total fibrinogen concentration in the plasma was measured before refrigeration. Plasma and serum samples were kept at 4 ℃ for up to 72h prior to assay of test substances. This was to allow precipitation of any abnormal fibrinogen into CF. Tubes were further spun at 1500g in a refrigerated centrifuge at 4 ℃ for 30 min. The supernatants were then rewarmed to 37 ℃ and used to measure fibrinogen and CF concentrations. The concentration of CF was measured as concentration differentials of fibrinogen before and after refrigeration.[22,23] The fibrinogen concentration was measured at 24 and 72 h after refrigeration. Sera samples were used for the detection and measurement of cryoglobulins (if present).

Assays of trypsin inhibitor and trypsin antibody

Trypsin inhibitor and trypsin inhibitor antibody (TI-Ab) in plasma and in CF[14] were measured by enzyme-linked immunosorbent assay (ELISA) in 18 patients with IBD (10 with UC, 8 with CD) as previously described.[24] These subjects were selected at random among the patients.

Ethics

All participants provided informed consent to be included in this study after they were informed of the purpose of the study and the nature of the procedures involved. Likewise the study protocol was reviewed and approved by our hospital Committee on the Ethics of Clinical Investigations Involving Humans.

Statistical analysis

Numerical data were presented as the mean ± SD values. Comparisons were made with the Chi square test (χ^2). Correlations between CF, TI and acute phase reactants including C-reactive protein (CRP), white blood cell counts (WBC), and erythrocyte sedimentation rate (ESR) were assessed by simple regression analysis or as indicated otherwise. $P<0.05$ was considered statistically significant.

RESULTS

Incidence and concentration of CF among groups

There was no significant difference in CF concentrations between 24 and 72h refrigeration. Therefore, only the results of 72h refrigeration were presented. A very high incidence of CF (upon storage of plasma at 4 ℃) and high CF concentrations were observed in patients with active IBD (Table 1 and Figure 1). This was most striking in patients with active UC (Figure 1). However, patients with UC who had operations or patients with non-IBD acute colitis (associated with infection) did not have abnormally high CF levels. Indeed, amongst the patients groups, the lowest CF concentration was found in patients with acute colitis; none of the 10 cases was CF positive. Thus in patients with UC, CF was still positive in 55.6% of patients with remitted UC, while patients with active CD had high CF levels, but the level in patients with inactive CD was not high (Figure 1). The only other group with high CF was patients with collagen disease, which is consistent with the literature[25]. In the disease control group, only 40% (12 of 40 patients) were positive for CF. In the healthy control group, only 1, (a 50-year-old man) was positive for CF, but the concentration of CF was not very high in this

Table 1 Plasma cryofibrinogen (CFG), fibrinogen levels and CFG/fibrinogen ratio in 284 subjects who participated in this study. When appropriate, the mean ± SD values are presented

Demography	UC			CD		Disease control			Healthy control
	UCA (n = 61)	UCR (n = 63)	UCPost-OP (n = 45)	CDA (n = 35)	CDR (n = 20)	Acute colitis (n = 10)	Collagen disease (n = 15)	Other diseases (n = 15)	(n = 20)
Age(yr)	35±19	33±12	36±17	32±11	27±11	34±11	38±12	33±16	34±18
Gender(M/F)	30/31	31/32	23/22	19/16	12/8	6/4	8/7	6/9	10/10
CFG Positive(%)	87.8[1a]	55.6[2]	33.3[3]	65.7[2b]	35.0[3]	0.0	66.7[2b]	13.3[3]	5.0
Fibrinogen (mg/dL)	318±109	240±96	303±66	287±137	251±66	254±55	384±107	206±96	246±59
CFG/fibrinogen ratio(%)	18±5[1a]	17±7[1a]	4±3	9±4[2]	4±3	0	7±4[2]	6±3[2]	2.5±0.7

UC: ulcerative colitis; UCA: active UC; UCR: remitted UC; CD: Crohn's disease; CDA: active CD; CDR: remitted CD; M: male; F: female; CFG positive (%): percentage of patients in whom cryofibrinogen was detected upon storage of plasma at 4℃, [1]χ^2<0.001 for active UC *vs* healthy control or other disease control; [2]χ^2<0.01 UCR and CDA *vs* healthy control or other disease control; [3]χ^2<0.05 UC Post OP, CDR *vs* healthy control or other disease; [a]χ^2<0.05 for UCA or UCR *vs* UCPost0OP, CDR, or other disease; [b]χ^2<0.05 for CDA, Collsgrn disease *vs* UCPost-OP, CDR, or other disease control.

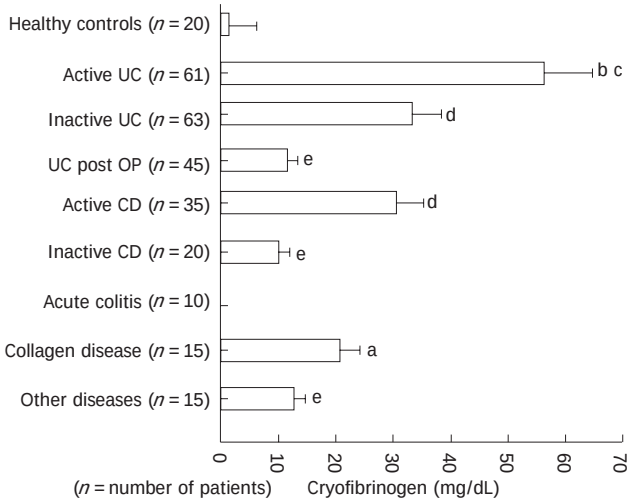

Figure 1 Cryofibrinogen (CF) concentration in subgroups of 284 patients. [b]*P*<0.001 for active UC *vs* healthy control or UC post OP, inactive CD, acute colitis, and other disease control; [d]*P*< 0.01 for inactive UC and active CD *vs* healthy control, UC post OP, inactive CD, acute colitis, and other disease control; [a]*P*<0.05 for collagen disease *vs* healthy control or UC post OP, inactive CD, acute colitis; [c]*P*<0.05 for active UC *vs* inactive UC and active CD; [e]*P*<0.05 for UC post OP, inactive CD, acute colitis, and other disease control *vs* healthy control.

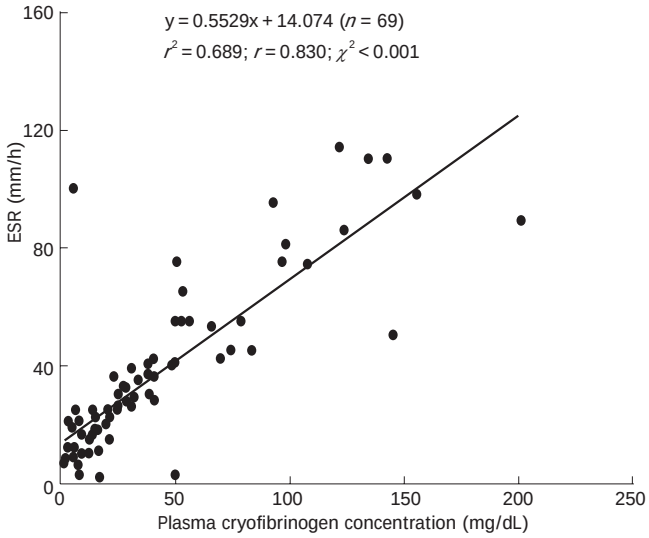

Figure 2 Simple regression analysis for relationship between plasma CF concentration and erythrocyte sedimentation rate (ESR) in 69 patients with active IBD.

patient (15.6 mg/dL). The CF/fibrinogen ratio was also determined for all subgroups of 284 patients. The results are presented in the Table 1. No cryoglobulinaemia was detected in IBD patients or in the healthy control group.

Relationship between CF and acute phase markers

To verify that high plasma CF was not associated with acute inflammation, its concentration was compared with concentrations of acute phase reactants like CRP, ESR, and WBC. Among these, only ESR showed a strong correlation with CF. (r=0.830, χ^2<0.001, Figure 2). There were no significant correlation between CF and CRP or between CF and WBC.

Relationship between CF and IBD clinical course

The relation between CF concentration, severity and duration of IBD were investigated. We divided the patients into 4 subgroups: (1) with more than 50 mg/dL CF; (2) with 10 to 50 mg/dL CF, (3) with less than 10 mg/dL CF; (4) with no CF. Both CAI/CDAI values and the duration of IBD were in this order: 1>2>3 or 4. We followed 44 patients who achieved remission among 61 with active UC and measured CF concentration to see if it follows patients' UC clinical course. Seven patients who became CF negative maintained their remission for over 5 years. In 33 patients in whom, CF concentration decreased by about 50%, 13 relapsed within 12 mo in addition to 4 patients who did not show any change in CF concentration.

Relationship between TI-ab and CF

It is thought that in the plasma, part of the CF complex acts as TI-ab and at 37℃ (the measurement temperature used in this study) and TI-ab dissociates from CF. Accordingly, TI-ab shown in both Figures 3 and 4 are believed to come from the CF complex. This means that a high plasma level of CF can reduce plasma levels of free TI. Figure 4 shows a simple regression analysis for the relationship between plasma CF and TI-ab in patients with

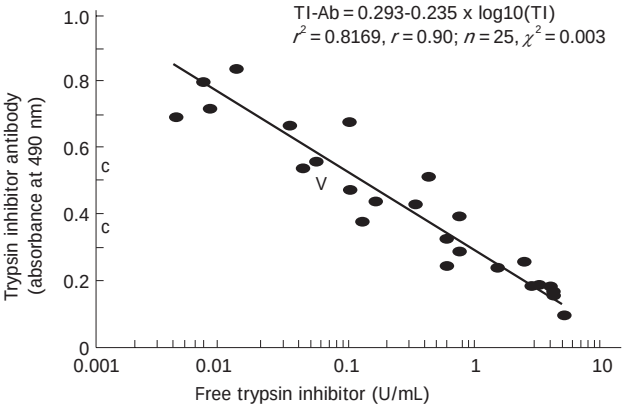

Figure 3 Logistic regression analysis to determine the relationship between TI and anti-TI-ab in patients with IBD.

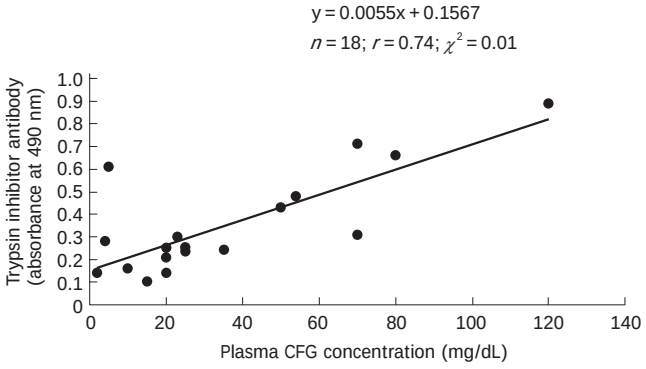

Figure 4 Simple regression analysis for the relationship between plasma CF concentration and TI-ab in 18 patients with active IBD.

active inflammatory bowel disease. As shown, there was a positive correlation between TI-ab and CF in the plasma (r= 0.74, χ^2 = 01).

DISCUSSION

For the first time this study reports the elevated plasma CF in patients with active IBD, with active UC showing the highest plasma CF. We also found that in most patients with active IBD, a marked fall in plasma CF was associated with disease remission. In contrast, persistently elevated CF was associated with medication-resistant and poor prognosis. Further, we could confirm that elevated plasma CF was not associated with typical acute phase reactants like CRP or WBC except showing strong correlation with the inflammatory marker, ESR. To ascertain that high plasma CF was one manifestation of active IBD, we included patient groups with diverse diseases together with a control group. Even in patients with acute colitis of non-IBD type, the concentration of CF was markedly lower compared with active UC of IBD. This observation convinced us that high plasma CF was indeed specific to active IBD. Further, elevated plasma CF was associated with IBD severity. Our impression is that elevated plasma CF is morbigenous; patients have severe and drug-refractory IBD.

Given that high plasma CF is seen during active IBD, and increases with IBD severity, the fundamental question if unequivocally addressed could significantly improve our understanding of the role of CF in IBD and the aetiology of IBD. First, is CF one cause or a consequence of active IBD? Second, exactly how CF contributes to active IBD (if at all)? At present, our data suggest that CF quenches TI thus depriving the immune system of an important natural anti-inflammatory factor (see the introduction section). But we should not forget that CF by precipitating cryoprotein aggregates is a major risk factor for vascular occlusion and impaired microvascular perfusion, which in IBD can delay ulcer healing.[3-8, 20, 25, 26] Additionally, high plasma concentration of CF can activate platelets in the same way as high plasma fibrinogen does[24] and block small vessels with micro-thrombi whereby compromising microcirculatory blood flow within the inflamed intestinal mucosa. Therefore, one mechanism of action of CF and its contribution to tissue injury and the perpetuation of IBD might be via vascular occlusion.

There are reports that treatment with immunosuppressive agents including corticosteroids, azathioprine, and cyclophosphamide is unsuccessful in preventing disease progression (non-IBD)[25-27]. However, the introduction of regular plasmapheresis together with cyclophosphamide therapy depletes plasma CF and this is associated with a marked improvement in the patients' clinical symptoms, Suggesting that extracorporeal circulation therapies such as plasma exchange and plasmapheresis, or cryofiltration[28-30] combined with conventional drug therapy may be effective in patients with refractory UC or CD who present with high plasma concentrations of CF, which we believe is a morbigenous factor in IBD.

Excess CF probably arises either from a misdirected synthesis or from post-synthetic alterations.[25,30,31] In IBD, it is likely that abnormal fibrinogen (CF) reflects a congenital abnormal production or an acquired abnormal production by the liver that is exposed to high cytokinaemia and proteases through portal vein from the intestine. If the elevated CF is caused by pro-inflammatory cytokines and proteases then its concentration in the inflamed intestinal lesions will be much higher compared with plasma.

In conclusion, high plasma CF in patients with active IBD appears to be morbigenous. The elevated plasma CF does not seem to be associated with typical acute phase reactants like CRP and WBC but showing strong correlation with ESR. Currently, our impression is that elevated CF promotes IBD via two main mechanisms quenching of TI (an anti-inflammatory substance) and impaired microvascular perfusion by forming protein aggregates. Further, CF may also serve as a new biomarker of chronic IBD. Clearly additional studies are warranted to fully evaluate the role of CF in IBD and the outcome should contribute to a better understanding of IBD aetiology.

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RAPID COMMUNICATION

Using p53-immunostained large specimens to determine the distal intramural spread margin of rectal cancer

Zhi-Zhong Pan, De-Sen Wan, Chang-Qing Zhang, Jian-Yong Shao, Li-Ren Li, Gong Chen, Zhi-Wei Zhou, Fu-Long Wang

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Abstract

AIM: To determine the distal intramural spread (DIS) margin of rectal cancer.

METHODS: Sixty-one p53-positive specimens of rectal cancer were used. After conventional hematoxylin and eosin (H&E) staining, the DIS margin of rectal cancer in large specimens was examined by immunohistochemistry. The patients were divided into A, B, C, and D groups. After a long-term follow-up, the survival curves of the four groups were estimated using the life table.

RESULTS: Fifty-one of the sixty-one cases (83.6%) had DIS. The extent of DIS ranged 0.11-3.5 cm; meanwhile the mean of DIS measured by H&E staining was 0.13 cm. The significant difference was found between the means (t=5.622, P<0.0001). Only 1 of 51 patients had DIS greater than 3 cm. The DIS was less than 1.0 cm in most rectal cancer patients. The long-term results indicated that the survival rate of the patients whose DIS was greater than 1.0 cm was lower than that of the patients whose DIS was less than 0.5 cm.

CONCLUSION: Rectal cancer patients with DIS greater than 1.0 cm have poor prognosis.

Key words: Rectal cancer; Distal intramural spread; p53; Immunohistochemistry; Large specimen

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INTRODUCTION

Defining the optimal distal surgical margin is very important for surgical oncologists during rectal cancer resection[1]. To ensure complete excision of cancer and maximum protection of normal tissue, we should define the length of DIS. Though a distal margin greater than 5 cm is advocated in the past[2], some studies proposed that a distal excision margin of 1 cm is sufficient[3-5], but the molecular clearance margin of DIS is unknown. Molecular techniques have been used to identify tumor markers and occult tumor cells in recent years[6] and the clear margin determined by molecular methods should be different from that by traditional histopathological methods. The accurate assessment of DIS could guide precise surgical resection, aid in estimating prognosis and predict recurrence site, but the molecular margin of DIS of rectal cancer is unknown. In this study, we used p53-immunostained large specimens of rectal cancer to measure the molecular margin of DIS and to clarify the influence of DIS margin on the long-term survival of patients after the resection of rectal cancer.

MATERIALS AND METHODS

Patients

By using conventional hematoxylin-eosin (H&E) and p53-immunostaining (LSAB), we identified 61 p53-positive cases in 97 rectal cancer patients who were surgically treated in our hospital between August 1996 and October 1997. The characteristics of the 61 patients are listed in Table 1.

Preparation of large specimens

Fresh specimens were opened longitudinally, straightened without stretching and pinned to a cork board. The distal margin (A1) was measured and fixed by 10% formalin for more than a week. The whole specimen including oral and anal edges of the tumor was cut longitudinally into 5-mm-thick giant sections. The sections were embedded in paraffin wax and then serial 8-μm-thick sections were cut from the giant sections on a large microtome. Routine H&E staining and p53-immunostaining (LSAB, 1:100 diluted mouse monoclonal antibody to p53, DO-7; DAKO Ltd, Denmark) were performed respectively. The distal margin was measured on large glass specimens (B1) and the extent of DIS was measured microscopically (b1). The microscopic extent of DIS was converted as the distance

Table 1 Clinicopathological characteristics of rectal cancer patients studied

Male	33
Female	28
Age (yr)	54 (22–79)
Gross morphology	
Fungating	14
Ulcerative	46
Infiltrative	1
Histologic type	
Papillary adenocarcinoma	8
Tubular adenocarcinoma	52
Mucinous adenocarcinoma	1
Histologic grade	
Well differentiated	8
Moderately differentiated	39
Poorly differentiated	14
Dukes' stage	
A	19
B	16
C	22
D	4

Table 2 Comparison of p53 immunostaining (IHC) and H&E staining in measuring the mean of DIS extents

DIS	IHC	H&E
0 cm	10	32
0.01 – 0.50 cm	26	26
0.51 – 1.00 cm	14	2
>1.00 cm	11	1
x	0.59 cm	0.13 cm
t	5.622	
P	<0.0001	

Table 3 Survival curves of groups A, B, C, and D

Group	Gehan value	*P*
A-B	1.204	0.273
A-C	1.685	0.194
A-D	5.359	0.021
B-C	0.814	0.367
B-D	4.627	0.032
C-D	1.124	0.289

of DIS in fresh specimen (a1) by the tissue shrinkage ratio by comparing the distal margin measured in fresh specimens before fixation (A1) to that measured on large sections macroscopically (B1) in each case (A1/B1=a1/b1). The data were converted data.

Statistical analysis

Both means of the DIS obtained by H&E staining and p53-immunostaining were compared by the paired sample statistic *t* test. According to the extent of DIS by p53-immunostaining, we divided the patients into four groups: A (DIS = 0.00 cm), B (DIS = 0.01-0.50 cm), C (DIS = 0.51-1.00 cm) and D (DIS > 1.00 cm) .The survival curves of the groups were generated by the life table and compared by Gehan test. SPSS software (8.0) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Modalities of DIS in rectal cancer

Under microscope, no cancer cells were found on the edge of resection in the large specimens. By p53 immunostaining, DIS of p53-positive tumor cells was found in normal glandular epithelial tissue next to the tumor in 23 cases under the mucosa away from the tumor in 40 cases. Emboli of cancer cells were found in micro-veins and/or micro-lymph vasculature under the mucosa in 35 cases. Nest formation of cancer cells was found under the mucosa away from the tumor and a clear line between the tumor and the normal tissue was noted in 21 cases. Spread of cancer cells through the above four pathways was observed but there was a clear large space between the metastatic cells and the main tumor.

Comparison between the extents of DIS by H&E staining and p53 immunostaining

By H&E staining, no DIS was found in 32 cases, 29 cases had DIS (range: 0.10-1.39 cm, mean: 0.13 cm, 95%CI: 0.1-0.16 cm). By p53 immunostaining, only 10 cases had no DIS, 51 cases had DIS (range: 0.11-3.5 cm, mean: 0.59 cm, 95%CI: 0.5-0.67 cm). There was a significant difference between the means obtained by p53-immunostaining and H&E staining ($t = 5.622$, $P < 0.0001$, Table 2).

Follow-up results

By December 2003, the follow-up time ranged 266-2 485 d with a mean of 1 621.4 d. All cases were followed up. Twenty-nine patients died of cancer recurrence. There were 32 disease-free cases in the study.

Survival analysis

The 5-year survival rate of 61 patients was 57.78%. The 5-year survival rate of groups A, B, C, and D was 79.41%, 60.93%, 55.70%, and 31.88%, respectively. There was no statistical difference among the survival rates of groups A, B, and C. The survival rate of group D was significantly lower than that of groups A and B (Table 3 and Figure 1).

DISCUSSION

Different from other studies, the whole specimen was expanded with a similar pull force by surgeons during operation and pinned on a flat board. Then the specimen was fixed by 10% formalin for more than 7 d. The extent of DIS was measured on large specimens microscopically. A tissue shrinkage ratio between the distance measured in fresh specimens from surgical margin to distal tumor edge was used to convert the extent of DIS microscopically to the extent of DIS *in situ*[7]. The error of DIS measured microscopically caused by the shrinkage of tissue in formalin may be avoided by the above method. Moreover, missed diagnosis of DIS should be reduced because the continuous intramural spread can be observed on large specimens.

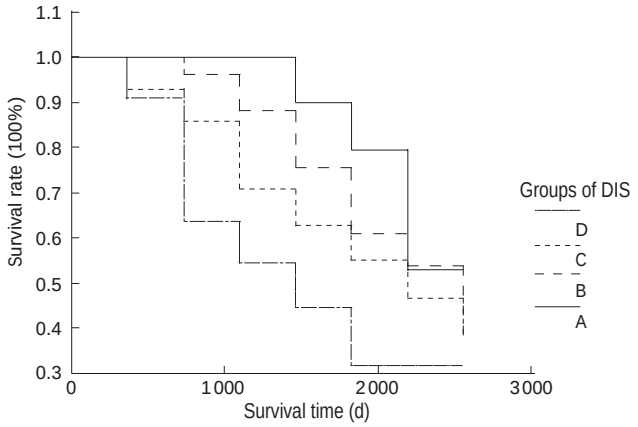

Figure 1 Survival curves of 61 rectal cancer patients.

Mutation of p53 gene is a late event of colorectal carcinogenesis[8]. Using molecular biological techniques, Brennan *et al*[9] and Hayashi *et al*[10] assessed the p53 gene mutation in clearance margins of 25 primary squamous cell carcinomas of the head and neck and local lymph nodes of 120 colorectal cancers. p53 gene mutation was found in some specimens without tumor residue, 40-70% patients with p53 gene mutation in clearance margins or lymph nodes had local recurrence and regional lymph node metastasis after the operation. No metastasis occurred in patients without p53 gene mutation. Their studies indicate that molecular biological analysis is more sensitive in finding hidden tumor cells by identifying tumor markers and can predict the site of local recurrence and the prognosis of patients.

Mutation of p53 gene results in the accumulation of p53 protein and p53 protein is regarded as a useful tumor marker[11]. p53-positive tumor cells that cannot be detected by H&E staining can be identified by immunohistochemical staining. Furthermore, hidden tumor cells usually are too small to be found by microscopy after conventional H&E staining.

In our study, we compared the extent of DIS by p53-immunostaining to that by H&E staining. The results between these two methods were similar, when DIS was less than 0.5 cm. In some cases, when DIS was greater than 0.5 cm, spread of single satellite cells in normal tissue was difficult to be identified by H&E staining. Therefore, H&E staining tends to cause false negative results compared to immune method. Because immunostaining is more sensitive than H&E staining, more positive diagnoses were made. The mean of DIS results was also higher by immunostaining ($P<0.05$, Table 2.).

Accurate identification of DIS of rectal cancer is very important for determining the clearance margin during the operation and preventing anastomosis recurrence[12]. According to the literatures published, DIS detected by H&E staining usually exists in 14.5-65.6% rectal cancer patients[3,7,8]. Because the results of DIS in rectal cancer are different in different studies[13], there is no universal agreement on the length of distal normal rectum that should be removed during radical resection[14]. Some authors suggested that 1-2 cm of distal removal of normal rectum is enough[4]. On the other hand, some cellular and molecular studies suggested that 2 cm is not safe[9]. In our study, though 83.6% (51/61) of the patients had DIS, the length of DIS in 78.4% (40/51) was less than 1 cm, in 6% (3/51) greater than 2 cm and only in 1.6% (1/51) greater than 3 cm, suggesting that 2 or 3 cm of the distal clearance margin is safe enough for most (95% or above) rectal cancers during radical resection.

It was reported that DIS is correlated to depth of cancer invasion and distal metastasis. Patients with positive DIS have a higher risk for developing metastasis after radical resection and their disease-free survival rate is lower than that of those with negative DIS[10]. In our study, patients with longer DIS had a lower survival rate (Figure 1). If DIS is greater than 1 cm, patient survival rate is markedly decreased regardless of distal normal rectum excision and negative tumor residue in distal surgical edge. The relationship between DIS and survival rate indicates that DIS is a prognostic factor of rectal cancer[15].

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CASE REPORT

Duodenal duplication cyst causing severe pancreatitis: Imaging findings and pathological correlation

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Abstract

We here report a case of a 18-year-old man with a history of recurrent abdominal pain and a previous episode of severe acute pancreatitis. Abdominal ultrasonography, contrast enhanced multislice computer tomography, endoscopic retrograde cholangiopancreatography, endoscopic ultrasonography and magnetic resonance imaging demonstrated a cystic mass lesion. Only on delayed phase magnetic resonance images after Gadolinium-BOPTA injection, it was possible to demonstrate the lesion's relationship with the biliary tree, differentiating the lesion from intraluminal duodenal diverticulum, and to achieve the diagnosis of duodenal duplication cyst, a recognized rare cause of acute pancreatitis. The diagnosis was confirmed by histology.

Key words: Pancreatitis; Congenital anomalies; Duodenal duplication cyst; Ultrasonography; Computed Tomography; Cholangiopancreatography; Magnetic Resonance Imaging

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INTRODUCTION

Duodenal duplication cyst (DDC) is a benign congenital anomaly acquired during the digestive system embryonic development. It is a recognized rare cause of acute pancreatitis, usually diagnosed in the first two infancy years. In adults, the diagnosis is difficult because of symptom variety and non-specific nature[1,2]. DDC represents 5-12 % of all gastrointestinal tract duplications and often communicates with either the small bowel or the pancreatic duct, rarely with the biliary system. Duplication anomalies are usually adjacent to the involved bowel. The morphology is spherical or occasionally tubular and may communicate with the lumen[1]. Moreover, they are composed of a smooth muscle wall and an inner mucosal lining. The type III choledochocele by Todani *et al*[3] is an isolated cystic dilatation of the distal portion of the choledochus, eventually protruding into the duodenal lumen, whose imaging findings are similar to those of duodenal duplication communicating with the bile duct[4,5]. The differential diagnosis between these two entities is often preoperatively impossible. Once the lesion is excised, only the different inner mucosa permits to perform a diagnosis. According to the magnetic resonance cholangiopancreatography (MRCP) findings, we report a case for which we suggest two possible final diagnoses: duodenal duplication cyst or type III choledochocele both communicating with the bile duct. In both computer tomography (CT) and endoscopic ultrasonography (EUS), previously performed, the relationship between the cystic lesion and the biliary tree was not clear. As far as we know, this is the first case where magnetic resonance (MR) with Gd-BOPTA (a contrast medium partially excreted by the biliary system) is able to demonstrate the relationship between the cystic lesion and the bile duct, which presents an anomalous pancreatic-biliary junction.

CASE REPORT

A 18-year-old man was referred to emergency room for an episode of severe acute pancreatitis. The patient was already known for previous recurrent abdominal pain. Laboratory values indicated an acute pancreatitis (increase of serum amylase and lipase activity). Other laboratory results including liver enzymes and peripheral blood count, serum protein and creatinine concentration were within the reference ranges. The patient did not have any primary cause of pancreatitis (e.g. alcohol consumption or choledocholithiasis). Ultrasonography (US) showed a hypoechoic area at the head of the pancreas, finding compatible with focal necrosis in pancreatitis. Contrast-

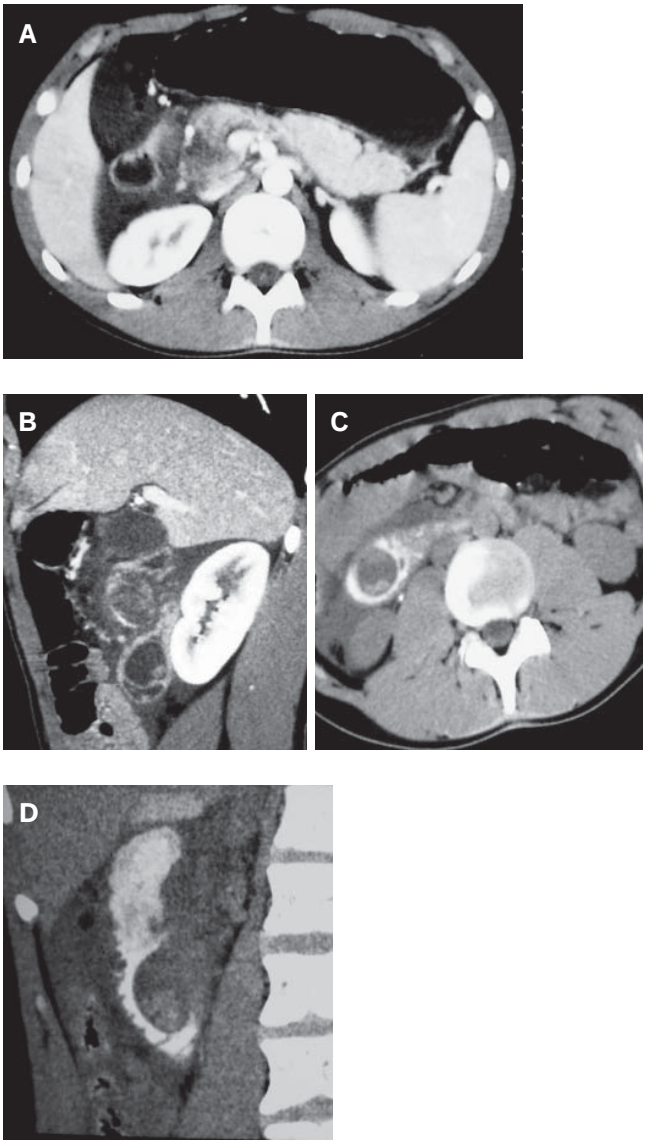

Figure 1 MSCT findings. **A** and **B**: On axial plane after i.v. injection of contrast medium the head of the pancreas presents a necrosis area, and on oblique plane the duodenum shows a cystic lesion filled with small stones; **C** and **D**: Oral contrast medium (diluted Gastrografin) fails to opacify the cystic lesion.

enhanced multislice CT (16 rows) confirmed the presence of necrotizing pancreatitis at the head (Figure 1A) and revealed a cystic lesion containing filling defects within the third portion of the duodenum (Figure 1B). After oral contrast agent (Gastrografin) administration, the cystic lesion was not filled up, suggesting the diagnosis of DDC (Figures 1C and D). The upper gastrointestinal series showed a four-centimeter intraluminal defect located in the medial wall between the second and the third portions of the duodenum. Endoscopic retrograde cholangiopancreatography (ERCP) was impossible to perform because the large submucosal mass was close to the major papilla, obstructing the bile ostium and pancreatic duct (Figure 2A).

EUS confirmed the presence of a submucosal cystic lesion at the inferior duodenal genu filled by calculi (Figure 2B), which was peculiar of choledochocele. Because of the site (duodenum medial wall) and the large size of the cystic

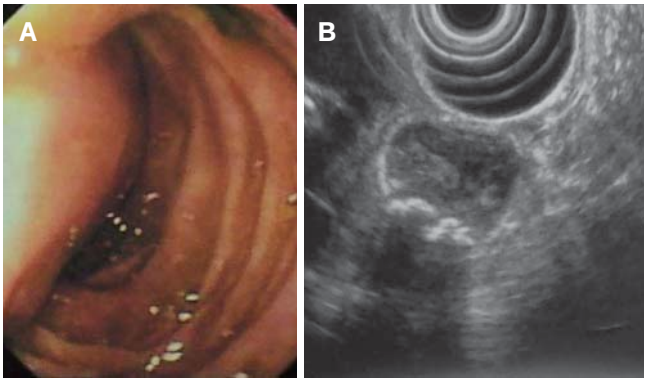

Figure 2 Endoscopic (**A**) and EUS (**B**) findings. By endoscopy the lesion appears as a submucosal mass obstructing the bile ostium and protruding into the duodenal lumen; EUS procedure was impossible to obtain further information because of the cystic lesion large size. The presence of calculi in the lesion was confirmed.

lesion it was impossible to demonstrate a linkage with the biliary system.

MR imaging was performed to solve the diagnostic discrepancy between CT and EUS and to better demonstrate the relationship between the cystic lesion and the bile duct. A 1.5 Tesla MR system with the following protocol was used: Breath Hold T1 GRE fat sat sequence (TR/TE 160/4,2), HASTE sequence TR/TE ∞/120 in the axial and coronal plane, RARE thick slab sequence (pre and post oral superparamagnetic contrast agent administration - Lumirem - Guerbet) and VIBE sequence in the coronal plane pre- and post-contrast agent administration (Gd-BOPTA), followed by a delayed phase at 120 min. The Gd-BOPTA is a mixed (interstitial and hepatocellular) paramagnetic contrast agent partially excreted through the biliary system, giving the opportunity like 99mTc-HIDA scintigraphy, to functionally evaluate the biliary excretion. All MR images showed a four-centimeter well circumscribed mass in the duodenal wall.

MRCP performed before and after a negative contrast agent oral administration, demonstrated that the cystic lesion was not directly in connection with the duodenal lumen. HASTE axial and coronal sequences, showed filling defects in the gravity-dependent position of the cystic lesion (Figure 3A). Heavily T2-weighted sequences (RARE thick slab) obtained in the coronal plane confirmed that the fluid content of the lesion was changing position with the peristalsis, without any definitive information about the relationship with the bile duct (Figure 3 B). Furthermore, on the delayed 3D VIBE images after intravenous (i.v.) injection of Gd-BOPTA, it was possible to demonstrate the direct passage of hyperintense bile fluid into the cystic lesion, confirming the existence of a linkage (Figure 4).

Once the patient recovered from the acute pancreatitis, one month after his first hospital admission, he underwent surgery. During laparotomy (Figure 5), after cholecystectomy and duodenotomy, two probes were inserted into the biliary tree through both cystic duct and ampulla: both the probes reached the diverticulum confirming the linkage between these anatomical structures. Then the diverticulum was opened, multiple stones were found and the wall

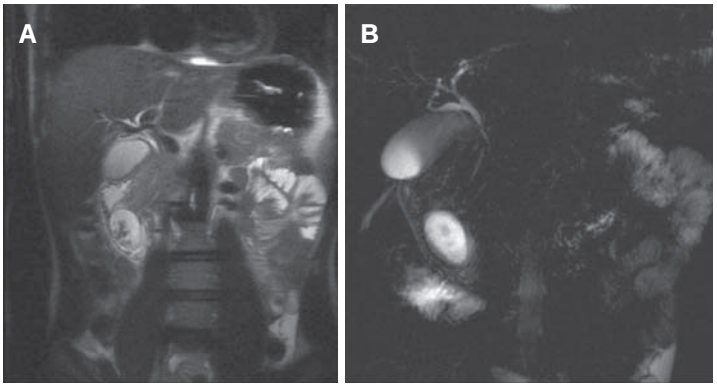

Figure 3 MRCP findings. **A**: Axial HASTE sequences demonstrate the intraluminal site of the duodenal cystic lesion with filling defects in the gravity dependent position; **B**: After superparamagnetic oral contrast medium administration the hyperintense cystic lesion does not change signal intensity.

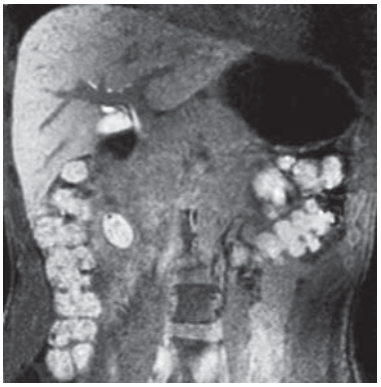

Figure 4 MRCP findings after Gadolinium BOPTA injection. Coronal VIBE sequences obtained in the delayed phase (2h) reveal the presence of hyperintense bile simultaneously in the biliary tree and in the cystic lesion, confirming the relationship between these two structures.

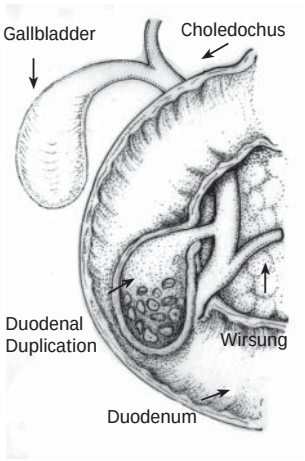

Figure 5 Schematic drawing of operative findings.

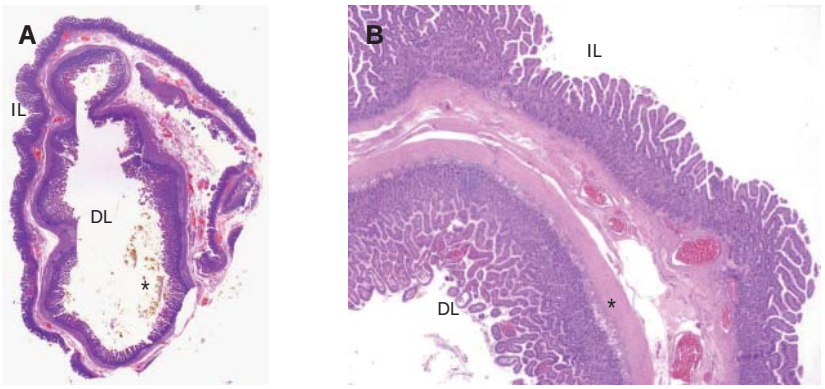

Figure 6 Mucosa, submucosa and muscle coats found in pathological ehamination. **A**: Gross transversal section of the specimen "in toto": inside it is possible to see the duplication lumen (DL) while the intestinal lumen is outside (IL). In the inner lumen there are bile plugs (*); **B**: Histology of the duodenal duplication. Microscopic low power view of the duplication wall (2x). The wall is composed of muscolaris propria extending into the septum between the duplicated segment and the mucosal lining. Intestinal mucosa is on both sides of the duplication, with a lot of macrophage cells (*) in the lamina propria of the internal one.

was completely excised up to the duodenal plane. The hole was closed with single layer stitches. Six days after surgery the patient was discharged.

Pathological examination of the excised cyst wall showed duodenal mucosa, submucosa and muscle coats. The pathologist made a diagnosis of duodenal duplication (Figures 6A and 6B) and found cholesterolosis in the cyst, in accordance with MR findings.

DISCUSSION

Clinical findings of DDC are either non-specific, such as mild abdominal pain, or specific, recalling acute or chronic pancreatitis. Two possible mechanisms might be responsible for pancreatitis: a transitory and mobility-related duodenal obstruction of the major papilla outflow by the cyst, and the migration of biliary sludge and/or microstones from the cyst to the biliary tree as observed in biliary pancreatitis[1]. As regards bile in- and out-flow, what we can imagine is a dynamic mechanism like gallbladder: the DDC wall is made of muscolaris propria, so there is peristalsis that outflows the bile. It is present a certain degree of stasis and demonstrated by the presence of stones. The pathogenesis of stones can be related more to the bile stasis in the cyst than to direct calculi drop down. Nevertheless the stasis, being intermittent due to peristalsis, does not automatically lead to a liver enzymes elevation, as in the literature[2,5,6]. The intraluminal mass has to be differentiated from a peduncled neoplastic lesion or an intraluminal duodenal diverticulum[6,7]. The cystic lesion is not an intraluminal duodenum diverticulum because the signal did not change after superparamagnetic contrast medium administration, remaining hyperintense in T2 weighted sequences. On opposite, the intraluminal duodenal diverticulum should become hypointense, due to the connection with the lumen. The differential diagnosis between these two different lesions is possible without administration of i.v. contrast medium[1]. Moreover MRCP alone cannot reveal the linkage between the cystic lesion and the bile duct, in particular if biliary anomalies are present. Following Gd-BOPTA injection, the presence of hyperintense biliary fluid on VIBE sequences can be demonstrated. The contrast agent fills both biliary tree and cystic duodenal duplication. In this case report the imaging findings of

duodenal duplication cyst communicating with the bile duct were comparable to that of choledochocele type III by Todani *et al*[3]. Considering the intraluminal site of the cystic lesion, it is possible to exclude other cystic diseases such as duodenal wall cystic dystrophy, pancreatic pseudocyst and other cystic masses belonging to the duodenal-choledochal-pancreatic area.

Regarding therapy, any surgical intervention should ensure complete resection of the duplication, in addition to its mucosa, in order to prevent malignant transformation[6].

CONCLUSION

Using both a negative oral contrast agent and an i.v. hepatospecific contrast agent with biliary excretion, MRI has a pivotal role in preoperative diagnosis to characterize duodenal cystic lesions eventually communicating with the biliary system and to differentiate duodenal duplication cyst from intraluminal duodenal diverticulum. In the case herein reported, distinction between choledochocele (type III) and duodenal duplication was only possible at pathological examination.

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CASE REPORT

Jumbo biopsy is useful for the diagnosis of colonic prolapsing mucosal polyps with diverticulosis

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Abstract

We report here a case of multiple prolapsing mucosal polyps with diverticulosis in the sigmoid colon. A 52-year-old man was admitted to our hospital because of bloody diarrhea. Colonoscopy and barium enema showed multiple diverticula, markedly thickened mucosal folds and polypoid lesions with mucus on the top of them in the sigmoid colon. Endoscopic ultrasonography showed thickening of the mucosal and submucosal layers. Several endoscopic biopsy specimens were taken from the polypoid lesions. Histological examination revealed only chronic inflammatory cell infiltration. In order to obtain a definite diagnosis, we performed endoscopic jumbo biopsy for the polypoid lesions after obtaining informed consent. Histological examination revealed marked lymphocyte infiltration, hemosiderin deposits and fibromuscular obliteration in the lamina propria, features similar to those of mucosal prolapsing syndrome. After anti-diarrhetic treatment, clinical findings were improved. Thus, jumbo biopsy is useful for diagnosis and treatment of prolapsing mucosal polyps.

Key words: Colon; Diverticulum; Jumbo biopsy; Prolapsing mucosal polyp

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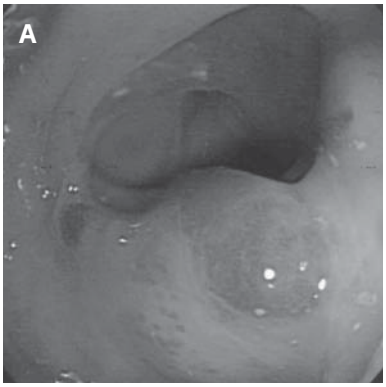
INTRODUCTION

Cases of colonic prolapsing mucosal polyps associated with diverticulosis have recently been reported in literature[1-3]. These polyps are histologically unclassifiable polyps resembling those in mucosal prolapse syndrome (MPS). Accurate differential diagnosis between neoplastic and non-neoplastic polyps is often difficult in these disease conditions. Many cases of colonic prolapsing mucosal polyps have been diagnosed retrospectively because of the low rate of pathological diagnosis of specimens obtained by the use of usual biopsy forceps. We report herein a case of unique non-neoplastic colonic mucosal prolapsing polyps with diverticulosis successfully diagnosed by using the jumbo biopsy method.

CASE REPORT

A 52-year-old man was admitted to our hospital in April 2003 because of abdominal discomfort, mucous discharge and bloody diarrhea. He had a history of frequent diarrhea from his twenties. Laboratory tests showed an increased number of peripheral leukocytes (13 800/mm^3). Barium enema showed polypoid lesions and multiple diverticula in the sigmoid colon. Colonoscopy showed multiple congested hyperemic mucosal folds and polypoid lesions with mucus on the top of them in the sigmoid colon. Slightly elevated red round patches were considered to be early minimal lesions of prolapsing mucosal polyps (Figure 1A). The surfaces of the polypoid lesions, considered to be advanced lesions, were smooth, and congested hyperemic mucosa contrasted sharply with the surrounding intact mucosa. Endoscopic examination with indigo-carmin dye spraying showed an intact mucosal pit pattern (Figure 1B). Endoscopic ultrasonography (EUS) of the early minimal lesions showed thickening of the first and second layers (mucosa) and irregular echoic lesions in the third layer (submucosa) with hypertrophy of the fourth layer (muscularis propria) (Figure 2A). EUS of the advanced lesions showed irregular low echoic lesions throughout the colonic wall (Figure 2B). Endoscopic



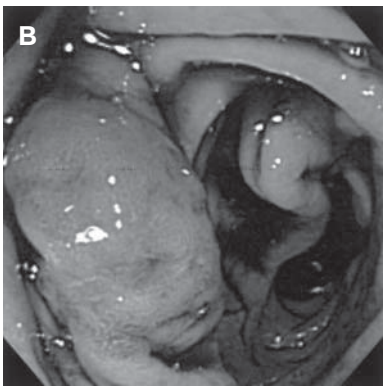
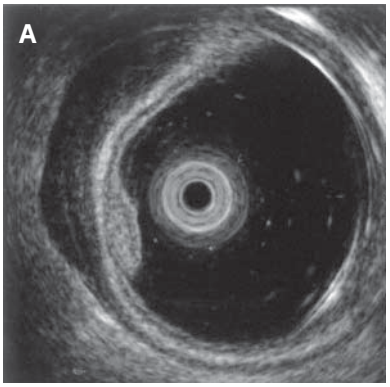


Figure 1 Colonoscopic examination of early minimal lesion (**A**) and advanced lesion (**B**) showing slightly elevated bright red round patches and congestive hyperemic polypoid lesion contrasted sharply with the surrounding mucosa (**B**: indigo carmine dye spraying view).



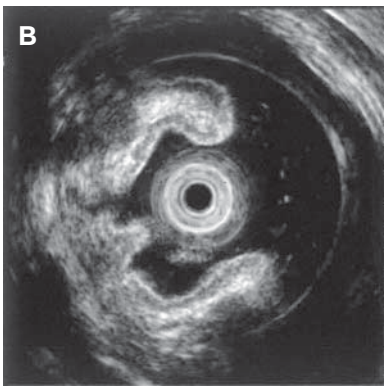
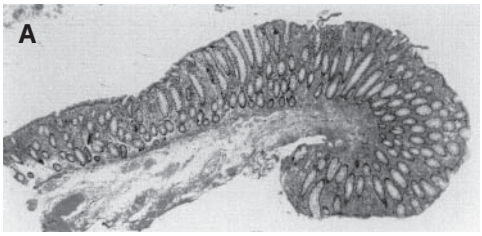


Figure 2 Endoscopic ultrasonographic examination of early minimal lesion (**A**) and advanced lesion (**B**) showing thickening of the first and second layers (mucosa) and irregular echoic lesion in the third layer (submucosa) with hypertrophy of the fourth layer (muscularis propria), and irregular low echoic lesion throughout colonic wall, respectively.



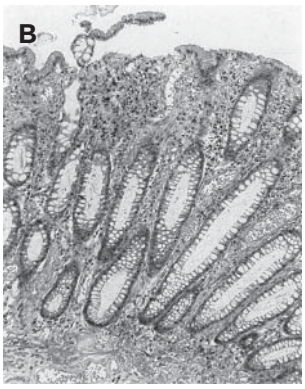


Figure 3 Hisological examination of prolapsing mucosal polyp showing mucosal hyperplasia (**A**) and chronic inflammatory cell infiltration, crypt elongation, congested vessels and fibromuscular obliterations observed in the lamina propria (**B**) (H&E, a: x40, b: x200).

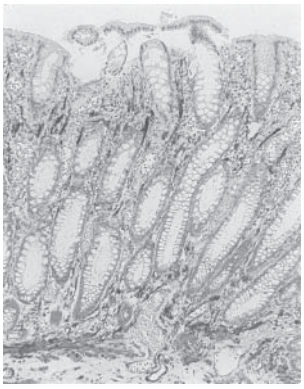


Figure 4 α-Smooth muscle actin (α-SMA) staining of prolapsing mucosal polyp showing smooth muscle fibers radiating from the thickened muscularis mucosa into the lamina propria (x200).

biopsy specimens were taken several times. Histology of biopsy specimens revealed no specific findings other than chronic inflammation. In order to obtain accurate diagnosis, we performed endoscopic jumbo biopsy for the polypoid lesions after obtaining informed consent. The jumbo biopsy specimen was taken by using a saline-solution-assisted snare resection technique that involved cutting with an Olympus SD-210-25 snare and an electro-surgical unit, OLYMPUS UES-20. Histology of the polypoid lesion showed mucosal hyperplasia (Figure 3A). Crypt elongation, chronic inflammatory cell infiltration, congested vessels and hemosiderin deposits were observed in the lamina propria (Figure 3B). Immunohistochemistry using a monoclonal antibody against anti-α smooth muscle actin showed smooth muscle fibers radiating from the thickened muscularis mucosa (fibromuscular obliteration) (Figure 4). Based on these findings, these lesions were diagnosed as prolapsing mucosal polyps with diverticulosis of the sigmoid colon. The patient was treated with anti-diarrhetic drugs and a fiber-enriched diet. Diarrhea was improved and the white blood cell count in peripheral blood decreased to within the normal range.

DISCUSSION

We experienced a patient who was diagnosed by using the jumbo biopsy method as having prolapsing mucosal

polyps with diverticular diseases. Cases of prolapsing mucosal polyps with diverticular disease have recently been reported by Franzin *et al*[1] and Mathus-Vliegen *et al*[2] These polyps are histologically unclassified polyps resembling those in mucosal prolapse syndrome (MPS). MPS including solitary rectal ulcer syndrome is characterized by the presence of polypoid or ulcerative lesions with the histological changes of glandular epithelium, fibromuscular obliteration and teleangiectatic blood vessels in the lamina propria. MPS is thought to result from chronic intermittent ischemia caused by overt or occult mucosal prolapse. Histological features of these polyps are crypt elongation, up-growth of muscle fibers from the muscularis mucosa (fibromuscular obliteration), and mucosal and submucosal vascular congestion, hemorrhage and hemosiderin deposition[3]. These polyps resemble 'cap polyposis'; however, there is a large amount of granulation tissue formation called fibrin-cap on the surface and less teleangiectactic changes in 'cap polyposis' than in MPS.

Endoscopy in our case showed slightly elevated red round patches, considered to be initial lesions, and smooth and congested hyperemic mucosal polypoid lesions. Tendler *et al*[4] reported that all prolapsing polyps occurred in the sigmoid colon usually in association with diverticular disease. Endoscopic ultrasonography (EUS) in our case showed thickening of the first and second layers (mucosa) and mixed hypoechoic lesions in the third layer (submucosa) with hypertrophy of the fourth layer (musclularis propria) in small patches, and moderate irregular hypoechoic lesions extended into the fourth layer (muscularis propria) with destruction of the first and second layers (mucosa) and the third layer (submucosa) in the polypoid lesion. It has recently been reported that EUS of polypoid prolapsing mucosal folds revealed thickening of mucosal and submucosal layers, demonstrating remarkably hyperemic and thickened mucosal folds[5]. These EUS findings resemble those described in previous reports of MPS[6,7]. In our case, the mixed hypoechoic pattern of early minimal lesions was thought to reflect mild fibromuscular obliteration and edema in the mucosal and submucosal layers. This irregular pattern was more apparent in advanced lesions throughout the colonic wall. These EUS findings demonstrated changes in the pathological development of these polyps. However, almost all cases of colonic prolapsing mucosal polyps have been diagnosed retrospectively after treatment. Only one case has been diagnosed by EUS findings and a normal surface pit-pattern before treatment[5].

Diagnosis of prolapsing mucosal polyps by examination of biopsy specimens obtained by using biopsy forceps can be difficult because of the sizes and depths of obtained specimens. Results of examination of biopsy specimens obtained several times from our case only showed chronic inflammation without fibromuscular obliteration. Warren *et al*[8] proposed the usefulness of diagnostic features of 'diamond-shaped' crypts in biopsy specimens. However, there were no such findings in our case. Jumbo biopsy is known to be a safe and useful method for diagnosis and treatment, and it is often used for not only endoscopic cure of early cancers, but also obtaining specimens for accurate pathologic staging[9]. In some cases, inflammatory change induces a "pseudo-malignant status". Colitis cystica profounda, a variant of mucosal prolapse syndrome, is sometimes difficult to distinguish from mucinous-producing carcinoma because of its formation by an aberrantly located gland-forming epithelium[10].

Treatment of prolapsing mucosal polyps is fundamentally conservative. Mathus-Viegen *et al*[2] reported the usefulness of a fiber-enriched diet for treatment of prolapsing mucosal polyps. Improvement was obtained in our case by a fiber-enriched diet and administration of an anti-diarrhetic drug. However, some patients with this disease require surgery because of intractable abdominal pain. It is important for endoscopists and pathologists to be familiar with and recognize the characteristic features of prolapsing mucosal polyps in order to avoid oversurgery for this disease because of its benign nature.

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CASE REPORT

Uveitis in autoimmune hepatitis: A case report

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Abstract

In this case report we describe for the first time an association between autoimmune hepatitis (AIH) and uveitis, without any doubts about other possible etiologies, such as HCV, since all the old reports describe the association of AIH with iridocyclitis before tests for HCV-related hepatitis could be available. A 38-year-old businessman with abnormal liver function tests and hyperemia of the bulbar conjunctiva was admitted to the hospital. Six years before admission, the patient presented with persistent fever, arthralgias, conjunctival hyperemia, leukocytosis and increased ESR, referred to acute rheumatic fever. The presence of systemic diseases, most commonly associated with uveitis, was investigated without results and the patient was then treated with topical corticosteroids. His symptoms resolved. A test for anti-nuclear antibodies was positive, at a titre of 1:320, with a speckled and nucleolar staining pattern. Liver ultrasound showed mild hepatomegaly with an increased echostructure of the liver. Percutaneous liver biopsy was performed under ultrasound assistance. Histological examination showed necroinflammation over the portal, periportal and lobular areas, fibrotic portal tracts, with periportal fibrosis and occasional portal-to-portal bridgings, but intact hepatic architecture. Some hepatocytes showed barely discernible granules of hemosiderin in the lobular area. Bile ductules had not any significant morphological alterations. METAVIR score was A2-F3, according to the modified HAI grading/fibrosis staging. The patient was diagnosed to have AIH with mild activity and fibrosis and was discharged on 25 mg prednisone, entering clinical and biochemical remission, further confirming diagnosis. After discharge the patient continued to have treatment with corticosteroids as an outpatient at a dose of 5 mg. On January 2002 the patient was readmitted to the hospital. A test for anti-nuclear antibodies was positive, at a titre of 1:320, with a speckled and nucleolar staining pattern. Anti-smooth muscle antibody test was also positive (1:160), while anti-LKM antibodies were negative. Ophthalmologic examination revealed inflammatory cells and proteinaceous flare in the anterior chamber of the left eye, and a stromal lesion in the cornea. He was maintained on immunosuppressive therapy (5 mg prednisone plus topical antibiotic therapy for two weeks) and then discharged. A complete remission of the symptoms was registered on follow-up. At present (July 2005), the patient is on prednisone (5 mg) and has no symptoms. Liver function tests are also within the normal range.

Key words: Autoimmune hepatitis; Uveitis

Romanelli RG, La Villa G, Almerigogna F, Vizzutti F, Di Pietro E, Fedi V, Gentilini P, Laffi G. Uveitis in autoimmune hepatitis: A case report. *World J Gastroenterol* 2006; 12(10): 1637-1640

http://www.wjgnet.com/1007-9327/12/1637.asp

CASE REPORT

A 38-year-old businessman was admitted to the Azienda Ospedaliero-Universitaria Careggi (Florence, Italy) on May 4th 2000, because of abnormal liver function tests and hyperemia of the bulbar conjunctiva.

The patient had no history of liver disease or jaundice, receipt of blood products, intravenous drug abuse, and exposure to alcohol or hepatotoxic drugs, seafood ingestion, any abdominal pain, excessive fatigue, rashes, or foreign travels. All his children were healthy. Family history of gastrointestinal or liver diseases was negative. His mother was affected by goitre and diabetes mellitus.

Six years before admission, the patient presented with persistent fever, arthralgias, conjuntival hyperemia, leukocytosis and increased ESR, which were referred to acute rheumatic fever, despite no evidence of recent group A streptococcal infection, since throat cultures were negative and streptoccal antibody test was normal. The patient was given salicylic acid up to 4 g for 2 mo. and prophylaxis with intramuscular benzathine penicillin G (1.2 g/mo. for 5 years). In 1998, conjunctival hyperemia recurred and iridocyclitis was diagnosed. The presence of systemic dis-

Table 1 Autoimmune hepatitis: Revised scoring system (1999) (International Autoimmune Hepatitis Group, *J Hepatol* 1999; 31: 929-938. Score Autoimmune Hepatitis)

	May 2000	January 2002
Gender: male	0	0
ALP/AST: 249/960 = *0.25* (admission May 2000); *3.85* (admission Jan 2002)	+2	-2
ALP/AST: 249/160 = *1.55* (discharge May 2000)	0	
ALP/ALT: 249/1 510 = *0.16* (admission May 2000); *4.15* (admission Jan 2002)	+2	-2
ALP/ALT: 249/483 = *0.51* (discharge May 2000)	+2	
IgG Assay: 1 590 (May 2000); 1 670 (January 2002)	+2	+2
ANA, SMA, or LKM1: negative	0	0
AMA: negative	0	0
Hepatitis markers: negative	+3	+3
Drug history: negative	+1	+1
Alcohol intake: negative	+2	+2
Histology: *Chronic hepatitis with moderate activity. Fibrotic portal tracts, periportal fibrosis with portal-portal septa, but intact architecture. Biliary ducts without any significant morphological alterations.*	+3	+3
Response to therapy: complete	+2	+2
Other autoimmune diseases: (thyroiditis)	+2	+2
Score:	21 (definite)	11 (probable)

eases, most commonly associated with anterior uveitis, was investigated without results and the patient was treated with topical corticosteroids. His symptoms resolved. At admission for further studies on systemic alterations other than uveitis, the temperature was 36.6 ℃, the pulse was 84 bpm, and the respiration rate was 18/min. Blood pressure was 105/70 mmHg. At physical examination, the head, the neck and the extremities were normal. Auscultation of the heart and both lungs were normal. The abdomen was soft with normal bowel sounds and not distended; there was tenderness in the right upper quadrant, and also in the lower one, with rebound tenderness in the latter; the liver was enlarged (about 3 cm below the right costal margin); the spleen was not palpable; no hernia was detected. There was no evidence of edema, bleeding diathesis, or spider angiomata.

Main initial laboratory findings were as follows: hematocrit 48.2%, leukocytes 5 400/μL (39.5% neutrophils, 39.7% lymphocytes, 18.2% monocytes, 0.6% basophyls, 2.0% eosinophils), platelets 157 000/μL, reticulocyte count 1.35%, glucose 0.83 mg/dL, BUN 0.25 mg/dL, creatinine 1.0 mg/dL, proteins 72 g/L, albumin 42.3 g/L, globulins 29.7 g/L, IgG 1 590 mg/dL, IgA 224 mg/dL, IgM 83 mg/dL, AST 960 IU/L, ALT 1 510 IU/L, γ-glutamiltranspeptidase 112 IU/L, alkaline phosphatase 249 IU/L, total bilirubin 1.57 mg/dL, conjugated bilirubin 0.4 mg/dL, total cholesterol 116 mg/dL, serum calcium 8.4 mg/dL, serum phosphorus 2.6 mg/dL, serum iron concentration 276 μg/dL, serum ferritin 3 020 ng/mL, and haptoglobin 54 mg/dL; international normalized ratio for prothrombin time was 1.3, the activated partial thromboplastin time 39.6 s, and TSH 2.22 mU/L. Urinanalysis was normal. Hepatitis B surface antigen, anti hepatitis A, B and C virus (IgG) were undetectable. Anti-CMV IgG were positive (30 UA/mL), but IgM resulted negative. Serologic tests for brucella and mycobacterium sp (IgG and IgM) were negative. The direct and indirect Coombs tests were also negative. Serum circulating immune complexes were within the normal range (IgG binding C1q 2.6 μEq/mL and IgG binding C3 5.2 μEq/mL), C3 was 111, C4 22 mg/dL. A test for anti-nuclear antibodies was positive, at a titer of 1:320, with a speckled and nucleolar pattern of staining. Test for anti-smooth muscle antibodies was also positive (1:160), anti-LKM antibodies were negative. Titres of antithyroid peroxidase autoantibodies and anti-thyreoglobulin autoantibodies were 420 and 85.6 IU/mL, respectively.

Liver ultrasound showed mild hepatomegaly with an increased echostructure of the liver. Percutaneous liver biopsy was performed under ultrasound assistance. Liver biopsy showed necroinflammation over the portal, periportal and lobular areas, fibrotic portal tracts, with periportal fibrosis and occasional portal-to-portal bridgings, but intact hepatic architecture. Some hepatocytes showed barely discernible granules of hemosiderin in the lobular area. Bile ductules were without any significant morphological alterations. METAVIR score resulted A2-F3, according to the modified HAI grading and fibrosis staging as proposed by Ishak *et al*[1]. No stainable iron or copper deposits were found.

Ophthalmologic examination, performed to rule out Wilson's disease, revealed no diminished visual activity in both eyes; eye movements were normal, without diplopia; the globes were intrinsically normal, so as papillary light reflexes. The fundus oculi was normal, without signs of vasculitis. Biomicroscopy of the anterior segment of the eye was negative for iridocyclitis. No Kayser-Fleischer rings were noted.

Considering the clinical presentation, together with increased serum aminotransferase levels, absence of viral markers for hepatitis B, C, and other hepatotropic viruses, evidence on liver biopsy of chronic hepatitis with mild-

Table 2 Extrahepatic associations of AIH

Frequent:	Autoimmune thyroid disease	Rare:	Rheumatoid arthritis
	Ulcerative colitis		Lichen planus
	Synovitis		Diabetes mellitus
	Autoimmune		CREST syndrome
	Hemolytic anemia		Autoimmune
			Thrombocytopenic purpura
			Vitiligo
			Alopecia

to-moderate fibrosis, the patients was diagnosed to have autoimmune hepatitis (AIH) with mild activity and discrete fibrosis. Patient was discharged on prednisone 25 mg and entered clinical and biochemical remission, further confirming diagnosis. After the discharge the patient continued to have treatment with corticosteroids as an outpatient at a dose of 5 mg.

On January 2002 the patient was readmitted to the hospital because of fever (39 ℃), occurring especially in the evening. He also complained photophobia, and blurred vision in both eyes. The blood pressure was 110/65 mmHg, the pulse rate was 87 bpm, and the respirations were 18/minute. Physical findings were unmodified in respect to the previous readmission.

Laboratory investigations showed the following data: hematocrit 39.7%, leukocytes 9 690/μL (65% neutrophils, 19.3% lymphocytes, 14.5% monocytes, 0% basophyls, 1.2% eosinophils), platelets 497 000/μL, reticulocyte count 1.35%, glucose 1.22 mg/dL, BUN 0.24 mg/dL, creatinine 1.2 mg/dL, proteins 84 g/L, albumin 33.9 g/L, globulins 50.1 g/L, IgG 1 670 mg/dL, IgA 273 mg/dL, IgM 64 mg/dL, AST 14 IU/L, ALT 16 IU/L, γ-glutamiltranspeptidase 26 IU/L, alkaline phosphatase 52 IU/L, total bilirubin 0.54 mg/dL, conjugated bilirubin 0.14 mg/dL, total cholesterol 96 mg/dL, serum calcium 9.1 mg/dL, serum phosphorus 2.4 mg/dL, serum iron concentration 82 μg/dL, serum ferritin 263 ng/mL, haptoglobin 1 120 mg/dL, international normalized ratio for prothrombin time 1.0, and the activated partial thromboplastin time 26.1 s. Flogosis indexes (ESR, C-reactive protein, fibrinogen) were elevated. TSH was 1.57 mU/L. Urinanalysis was normal. Direct and indirect Coombs tests were both negative. Serum circulating immune complexes were within the normal range; C3 was 196 mg/dL, C4 43 mg/dL, and serum immunoelectrophoresis showed increased IgG. A test for anti-nuclear antibodies was positive, at a titre of 1:320, with a speckled and nucleolar staining pattern. Test for anti-smooth muscle antibodies was also positive (1:160), anti-LKM antibodies were negative. Titres of antithyroid peroxidase autoantibodies and anti-thyreoglobulin autoantibodies were 420 and 85.6 IU/mL, respectively.

Serologic markers of viral infection (toxoplasma, EBV, mycobacterium sp, CMV, HAV, HBV, HCV and neurotropic viruses) were all negative. Antistreptococcal and anti-staphylococcal antibodies were undetectable (Table 1).

Ultrasound of the liver was normal. Ophthalmologic examination revealed inflammatory cells and proteinaceous flare in the anterior chamber of the left eye (Tyndall + - -), and a stromal lesion in the cornea. The right eye was unaffected.

He was maintained on immunosuppressive therapy (5 mg prednisone) plus topical antibiotic treatment for two weeks and then discharged. A complete remission of the symptoms was registered on follow-up. At present (July 2005), the patient is on prednisone (5 mg) and has no symptoms. Liver function tests are also within the normal range.

DISCUSSION

About 25% of patients with AIH have an acute onset. In most cases, however, the clinical presentation of AIH is characterized by the same signs and symptoms of chronic hepatitis of other aetiology. A specific feature of AIH is the association with extrahepatic immune-mediated syndromes, including autoimmune thyroiditis[2-5], scleroderma, rheumathoid arthritis, Sjögren's syndrome or diabetes mellitus (Table 2). An old report describes the association of chronic autoimmune hepatitis with iridocyclitis[2], but at that time tests for HCV-related hepatitis were not available[6].

The hallmark of acute anterior uveitis is the presence of inflammatory cells and proteinaceous flare in the anterior chamber of the eye. Symptoms include pain, photophobia, and blurred vision in the involved eye(s). Iridocyclitis may be part of a more generalized autoimmune and endogenous uveitis, such as Reiter's syndrome, more commonly observed in patients with HIV infection. Phacogenic uveitis, sympathetic ophtalmia and Vogt-Kaganayi-Harada syndrome can represent ocular autoimmune diseases suitable for a differential diagnosis, even if both phacogenic uveitis and sympathetic ophtalmia are post-traumatic granulomatous uveitis. In this patient, however, there is no history of physical or surgical trauma, ruling out the possibility of an involvement of the above disorders in this case. The Vogt-Kaganayi-Harada syndrome is an idiopathic, bilateral, inflammatory syndrome occurring in middle age and characterized by a typical granulomatous intraocular inflammation. The aetiology of this syndrome is unknown and the disorder is assumed to be an autoimmune hypersensitivity response to pigment. Poliosis (i.e. localized depigmentation of the hair) occurs in more than 90 percent of these patients, together with alopecia and vitiligo. Auditory disturbances occur in more than 75% of patients, and many other neurologic findings, including psychosis, have been sometimes reported. However, this disorder is very rare, and most cases have been associated with a previous penetrating ocular injury, which is absent in our patient.

We also considered, for the differential diagnosis, a possible ophtalmic localization of systemic diseases, either infectious or not[7]. Wegener's granulomatosis is characterized by fever, weight loss and other systemic manifestations, together with ocular and oropharyngeal signs. Moreover, ankylosing spondylitis and chronic inflammatory bowel diseases can both be complicated by anterior uveitis. Furthermore, viral infections can cause uveitis: toxoplasmosis is generally the most common cause of infective uveitis, and AIDS is also mostly associated with cryptococ-

cal chorioretinitis or posterior uveitis. A possible cause of uveitis can, finally, be represented by systemic lupus erythematosus, which is typically characterized by arthralgia, fever and ocular involvement, the presence of circulating antinuclear autoantibodies and no substantial remission after corticosteroid therapy.

AIH type I is the most common form of AIH worldwide and is associated with antinuclear antibodies and/or smooth muscle antibodies. This is a disorder characterized by hepatic and extrahepatic involvement; the most frequent extrahepatic associations of AIH are mentioned in the table above *(*Table 2*)*. In this case report, we have described, for the first time, an association between AIH and uveitis, without any doubts about other possible etiologies, such as HCV infection[8], since all the old reports describe the association of AIH with iridocyclitis before tests for HCV-related hepatitis could be available. Furthermore, this report concentrates on clinical attention to patients who may be at increased risk for potentially serious ocular disorders and for whom slit-lamp examination may be warranted on a regular basis.

The majority of patients affected by AIH require a long-term maintenance therapy, and the milder disease is associated with a better response to therapy[9]. Corticosteroid treatment is the best choice therapy for AIH and, even if it is usually followed by immunosuppression, thereafter[10], we preferred a low-dose steroid treatment, registering both remission of hepatitis and absence of any significant side effects. This is well documented in the literature[11]. Moreover, azathioprine has a significant early adverse reaction (EAR) profile, which includes an acute syndrome of constitutional symptoms, fever, rash, and acute pancreatitis and often requires discontinuation of drug. Some Authors report that EAR (early adverse reactions) precludes azathioprine use in patients with Crohn's disease (CD) and autoimmune hepatitis (AIH)[12]. Finally, we skip immunosuppressive treatment since it has been described an increased tumor risk under azathioprine therapy, especially in Vogt-Kaganayi-Harada (VKH) syndrome. Vogt-Kaganayi-Harada (VKH) syndrome was excluded in the differential diagnosis of our patient. Azathioprine treatment has been registered associated with increased risk of malignancies[13,14].

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CASE REPORT

Dermatomyositis associated with hepatocellular carcinoma in an elderly female patient with hepatitis C virus-related liver cirrhosis

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Abstract

A 79-year-old female patient with hepatitis C virus-related liver cirrhosis was diagnosed as having hepatocellular carcinoma (HCC) with a diameter of 2.0 cm. She refused therapy for HCC. Nine months after the diagnosis, she developed dermatomyositis when the HCC enlarged to a diameter of 6.0 cm. She underwent therapy for dermatomyositis, and then transcatheter arterial chemoembolization for HCC. Although the manifestations of dermatomyositis improved and entire tumor necrosis was achieved, she died of pneumonia 2 mo after the treatment of HCC. HCC and/or chronic hepatitis C virus infection might be involved in the pathogenesis of dermatomyositis.

Key words: Dermatomyositis; Hepatocellular carcinoma; Hepatitis C virus

Toshikuni N, Torigoe R, Mitsunaga M, Omoto A, Nakashima K. Dermatomyositis associated with hepatocellular carcinoma in an elderly female patient with hepatitis C virus-related liver cirrhosis. *World J Gastroenterol* 2006; 12(10): 1641-1644

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INTRODUCTION

The cause of dermatomyositis remains unknown. However, some factors have been implicated in the pathogenesis of dermatomyositis. Many reports on the relationship between dermatomyositis and cancer or viral infection have been published[1-17]. We report herein a rare case of dermatomyositis that occurred during the natural course of hepatocellular carcinoma (HCC) in an elderly female patient with hepatitis C virus (HCV)-related liver cirrhosis.

CASE REPORT

A 79-year-old woman was treated for HCV-related liver cirrhosis at our hospital. She received medication for the liver disease (ursodeoxycholic acid) and for epilepsy (clonazepam) due to a past head injury. There was no family history of autoimmune disease or malignancy. In October 2003, a hepatic tumor with a diameter of 2.0 cm in the right anterosuperior segment was detected on dynamic computed tomography (CT): the tumor was shown to be a hypodense lesion in the basal study which was enhanced during the arterial phase and became hypodense during the delayed phase. The serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) levels were 3.4 ng/mL (normal, <10 ng/mL) and 431 mAU/mL (normal, <40 mAU/mL), respectively. Based on these findings, the tumor was diagnosed as HCC. However, the HCC was not treated at that time because the patient refused therapy.

In July 2004, she began to develop progressive muscle weakness in the extremities and systemic erythema. She was admitted to the Department of Dermatology at our hospital. On admission, her height was 146 cm and her body weight was 60 kg. A heliotrope rash in the periorbital skin, Gottron papules on the metacarpophalangeal joints, and poikiloderma on the anterior neck and upper chest were observed. There was no skin thickening of the extremities or joint swelling. Muscle examination revealed symmetrical proximal weakness: she could not rise from a chair or raise her hands up to the level of her shoulders. Table 1 shows her laboratory data on admission. Elevated serum muscle enzymes were observed. HBsAg (enzyme immunoassay [EIA], Dinabot, Tokyo, Japan) was negative. Anti-HCV antibody (second generation, EIA, Dinabot) was positive with a high titer, and HCV RNA (reverse transcription-PCR, Sionogi, Osaka, Japan) was also positive. Hypergammaglobulinemia was observed. Antinuclear antibodies were positive (1:640, homogeneous, speckled), while rheumatoid factor, myeloperoxidase antineutrophil cytoplasmic antibodies, anti-DNA, anti-

Table 1 Laboratory data on admission

			Range				Range
WBC	3190	/mm^3	3500-8500	CPK	2092	IU/L	46-160
RBC	401×10^4	/mm^3	380×10^4-480×10^4	aldolase	13	IU/L	2.5-6.2
Hb	13.7	g/dL	11.8-15.0	myoglobin	293	ng/mL	≤60
Plt	9.0×10^4	/mm^3	13.0-30.0×10^4				
PT	11.6	s	10.0-13.0	IgG	2027	mg/dL	870-1700
				CRP	0.08	mg/dL	0-0.4
AST	150	IU/L	≤35				
ALT	39	IU/L	≤35	HBsAg	(-)		
LDH	583	IU/L	≤230	anti-HCV	(+)		
ALP	200	IU/L	≤340	HCV RNA	(+)		
Γ-GTP	20	IU/L	≤55				
T-Bil	0.8	mg/dL	0.2-1.2	ANA	× 640		
ChE	192	IU/L	185-431		HO, SP		
T-CHO	170	mg/dL	≤220	RF	(-)		
TP	5.7	g/dL	6.5-8.0	MPO-ANCA	(-)		
Alb	2.7	g/dL	4.2-5.0	anti-Jo-1	(-)		
BUN	15.3	mg/dL	9.0-20.0	anti-DNA	(-)		
Cr	0.6	mg/dL	0.4-1.1	anti-RNP	(-)		
FBS	96	mg/dL	≤110	anti-SSA	(-)		
Ca	8	mg/dL	8.5-10.2	anti-SSB	(-)		

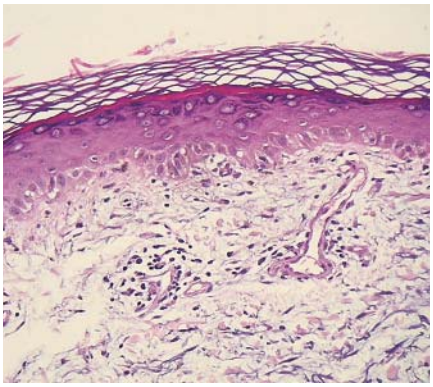

Figure 1 Hematoxylin-eosin staining of skin biopsy of the left forearm showing flattened epidermis and scattered inflammatory cell infiltration around the small vessels of the dermis (200 × magnifications).

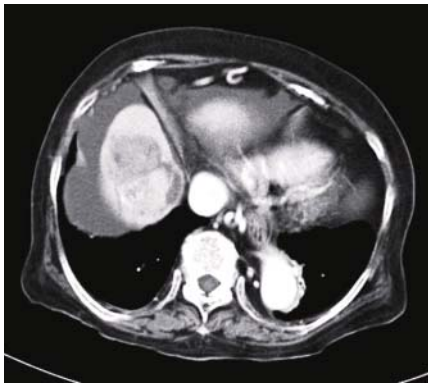

Figure 2 Contrast-enhanced computed tomography showing a hypervascular, in part, necrotic tumor in the right anterosuperior segment of the liver and ascites.

Jo-1, anti-RNP, anti-SSA, and anti-SSB were all negative. Electromyography of the right biceps brachii muscle showed fibrillation and low amplitude voltage of a short duration (less than 200 mV). Skin biopsy of the left forearm showed flattened epidermis, partial liquefaction of the basal layer, scattered inflammatory cell infiltration around the small vessels, and degeneration of collagen fiber of the dermis (Figure 1). Muscle biopsy of the left triceps failed to show remarkable findings. On the basis of these findings, the diagnosis of dermatomyositis was made. Initially, 50 mg of prednisolone per day was administered, and then the dosage was gradually decreased to 15 mg/d. Skin eruption almost disappeared completely and muscle power slowly improved. Muscle enzymes also improved to the normal range. During the treatment of dermatomyositis, the patient decided to undergo therapy for the HCC.

When she was referred to the Department of Internal Medicine in August 2004, the HCC nodule was enlarged to a maximal diameter of 6.0 cm, and ascites was observed (Figure 2). The serum AFP and DCP levels also increased to 2 130.2 ng/mL and 7 423 mAU/mL, respectively. Other serum tumor markers, including carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 125, were all negative. No other malignancies were identified by imaging studies, such as ultrasonography and CT or esophagogastroduodenal endoscopy. In August 2004, she underwent transcatheter arterial chemoembolization for the treatment of the HCC after the ascites was controlled. After transcatheter arterial chemoembolization, entire tumor necrosis was achieved; the serum AFP and DCP levels decreased to 46.7 ng/mL and 54 mAU/mL, respectively. The clinical course of dermatomyositis was not affected by the embolization. Unfortunately, the patient died of bacterial pneumonia in October 2004.

DISCUSSION

The relationship between cancer and dermatomyositis has been widely reported in the literature[1-9]. Recent

population-based studies have demonstrated that 15-32% of patients with dermatomyositis are associated with various types of cancers, such as ovarian, lung, pancreatic, stomach, and colorectal cancers[1,2]. However, there have been few reports on the association between dermatomyositis and HCC[3-9]. Although the rare association might be fortuitous, a recent report described the improvement of dermatomyositis without the need of corticosteroids after the resection of HCC, supporting the hypothesis that HCC can cause dermatomyositis through a paraneoplastic mechanism[8]. In the present case, dermatomyositis occurred when the HCC increased from a baseline diameter of 2.0 cm to 6.0 cm at 10 mo. In most other reported cases, advanced HCC, more than 6.0 cm in diameter or multiple, has been found at the diagnosis of dermatomyositis[4-9]. Those reports indicate that advanced HCC might induce an autoimmune response as the trigger of dermatomyositis.

Recent studies have demonstrated that antibodies to Mi-2, a component of the nucleosome remodeling-deacetylase complex, are strongly associated with dermatomyositis (frequencies up to 31%)[18,19]. An *in vitro* study has shown that a 169-bp cDNA product, which is 88.8% homologous to the human Mi-2beta antigen, is identified in H4IIE rat hepatoma cells[20]; and 100% homology is found at the protein level. Based on these findings, anti-Mi-2 antibodies might be cross-reactive with HCC. Unfortunately, anti-Mi-2 antibodies were not examined in the present case. Further studies on autoantibodies cross-reacting with HCC should be undertaken to shed light on the pathogenesis of dermatomyositis associated with HCC.

Occasional associations between dermatomyositis and HCV infection have been reported[6,7,9-14]. Thus far, there has been no direct evidence that HCV infection can cause dermatomyositis. A recent preliminary study has shown no significant higher incidence of HCV in patients with dermatomyositis as compared with the control population[10]. However, chronic HCV infection has been known to be associated with the presence of autoantibodies and various types of autoimmune diseases, leading to the hypothesis that chronic HCV infection could cause dermatomyositis through an autoimmune mechanism[21]. In the present case, antinuclear antibodies were strongly positive, suggesting that there might be some immune disorders related to the development of dermatomyositis. We speculate that antibodies against HCV or HCV-enzyme complex might cross-react with homologous area of host proteins and thus cause autoimmune diseases including dermatomyositis. Crowson *et al*[22] have reported that HCV RNA is expressed in a focal, weak fashion in endothelia and perivascular inflammatory cells in some HCV-infected patients associated with cutaneous eruptions. They postulated that parasitism by HCV can render endothelia autoantigenic through exposure of cryptic antigens via virally induced endothelia cell injury. Histopathologic examinations of HCV expression using skin biopsy specimens from patients with dermatomyositis associated with HCV infection might provide information on the role of HCV in the pathogenesis of dermatomyositis.

In the present case, other infectious agents including echovirus, adenovirus, and coxsackievirus, which are known to cause myositis, were not examined[15-17]. Therefore, there remains the possibility that dermatomyositis of the present case might be caused by such viruses.

In summary, this report suggests that HCC, in particular advanced HCC, and/or chronic HCV infection might be factors in the pathogenesis of dermatomyositis. The mechanisms of autoimmune responses induced by such factors should be studied in the future.

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CASE REPORT

Spontaneous regression of hepatic inflammatory pseudotumor with primary biliary cirrhosis: Case report and literature review

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Abstract

Hepatic inflammatory pseudotumor (IPT) is a rare benign non-neoplastic lesion characterized by proliferating fibrous tissue infiltrated by inflammatory cells. The exact etiology of IPT remains unclear. Although the association of IPT with systemic inflammatory disorders has been well established, a specific relationship with cholangitis is distinctly rare. We report a case of spontaneous regression of hepatic IPT with primary biliary cirrhosis (PBC). To date, only two cases of IPT with PBC have been reported. In our case, however, IPT developed during the course of improvement of cholangitis of PBC induced by effective treatment, differing from two previously reported cases. Our case indicates that the development of IPT does not also relate to the activity of cholangitis and/or hyper gamma-globulinemia, since our case was confirmed radiologically to be free of IPT when biliary enzymes and immunoglobulins were much higher than the corresponding values on admission. Comparison of our case with the two previously reported cases suggests that IPT occurring with PBC does not represent the same disease entity or be a bystander for PBC.

Key words: Hepatic inflammatory pseudotumor; Primary biliary cirrhosis; Spontaneous regression; Ursodeoxycholic acid; Bezafibrate

Koide H, Sato K, Fukusato T, Kashiwabara K, Sunaga N, Tsuchiya T, Morino S, Sohara N, Kakizaki S, Takagi H, Mori M. Spontaneous regression of hepatic inflammatory pseudotumor with primary biliary cirrhosis: Case report and literature review. *World J Gastroenterol* 2006; 12(10): 1645-1648

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INTRODUCTION

Hepatic inflammatory pseudotumor (IPT) is a rare benign non-neoplastic lesion characterized histopathologically by proliferating fibrous tissue infiltrated by inflammatory cells. The exact etiology of IPT remains unclear. Although the association of IPT with systemic inflammatory disorders has been well described, a specific relationship with cholangitis is distinctly rare[1-4]. Primary biliary cirrhosis (PBC) is a chronic cholestatic disease with cholangitis. We report a case of spontaneous regression of IPT associated with PBC. Our case was peculiar in view of detection of IPT during improvement of cholangitis of PBC, thus differing from the two previous reports of IPT with PBC[5,6].

CASE REPORT

The patient was a 71-year-old man. He was diagnosed in 2001 with PBC (Scheuer's histological stage II) based on histopathological findings of liver biopsy specimens and positive anti-mitochondrial antibody (AMA) and was followed with ursodeoxycholic acid (UDCA) in our department. He requested a check up for cancer and fluorodeoxyglucose positron emission tomographic study (FDG PET) was performed on his request on his 70th birthday. The whole-body images demonstrated a 3-cm-diameter spherical mass in the liver (Figure 1A, B). One year before FDG PET, no liver tumor was noted on enhanced computerized tomography (CT). He was admitted to our department for full examination and assessment. We had started treating the patient with bezafibrate, which was recently reported to be effective in improving PBC patients with elevated biliary enzymes[7], two months before detection of the liver mass because UDCA had not been fully effective. On admission, he was asymptomatic and physical examination was negative. Laboratory data on admission (Table 1) showed elevated

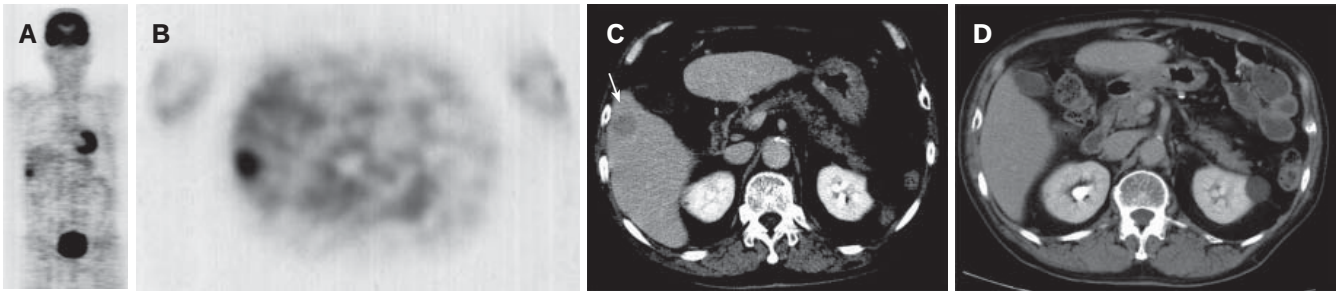

Figure 1 A, **B**: Whole body F-18 fluorodeoxyglucose (FDG) positron emission tomography (PET) scan showed a 3-cm spherical mass in the right lobe of the liver. **A**: Coronal sectional view, **B**: horizontal sectional view. **C**: Enhanced CT showed a 3-cm spherical low-density mass (arrow) in the right lobe of the liver. **D**: Another enhanced CT performed 3 mo after targeted liver biopsy showed complete resolution of the mass.

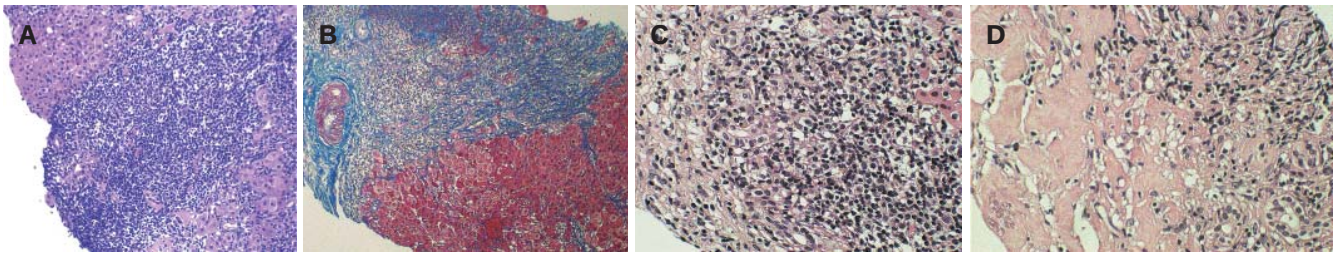

Figure 2 A: Liver biopsy specimen from the non-tumorous liver. Histopathological findings are consistent with primary biliary cirrhosis. The enlarged portal tract with damaged bile ducts in florid lesion of non-suppurative destructive cholangitis is infiltrated by inflammatory mononuclear cells. H&E, X100. **B**: Liver biopsy specimen shows clear-cut boundary between the "tumor" and liver parenchyma. Azan-Mallory, ×100. **C**:The "tumor" is composed of chronic inflammatory cells including lymphocytes and plasma cells. H&E, × 400. **D**: The "tumor" is composed of fibrous tissue, thick hyalinized collagen bundles with disappearance of liver parenchyma. H&E, ×400.

Table 1 Changes of Biochemical and immunologic Profile

	Before treatment of UDCA without detection of IPT	On admission	At the time of regression of IPT
Total bilirubin (mg/dL)	0.7	0.8	0.6
AST (IU/L)	46	22	25
ALT (IU/L)	391	16	15
ALP (IU/L)	682	159	141
γGTP (IU/L)	248	52	45
IgM (mg/dL)	1010	303	281
IgG (mg/dL)	2010	1360	1170
ANA	×5120	×160	×160
AMA (index)	181	205.6	173.2
CRP	<0.1	<0.1	<0.1

AMA (205.6 arbitrary unit), anti-nuclear antibodies (ANA, ×160), gamma-glutamyl transpeptidase (gamma-GTP, 52 IU/L) and IgM (303 mg/dL) levels and a normal value of alkaline phosphatase (ALP). The results of these tests showed satisfactory improvement compared with the data recorded when treatment was limited to UDCA only, thus reflecting the effectiveness of bezafibrate. Total bilirubin, aspartate aminotransferase, alanine aminotransferase, C-reactive protein, IgG levels, leukocyte count and gamma-globulin ratio of serum protein were normal. Serology for hepatitis B and C, alpha-fetoprotein, protein levels induced by the absence of vitamin K or antagonist-II and carcinoembryonic antigen were normal. Magnetic resonance cholangiopancreatography (MRCP) findings were normal. Enhanced CT showed a 3-cm spherical low-density mass in the right lobe of the liver (Figure 1C) corresponding to the mass on FDG PET. To rule out hepatocellular carcinoma, metastatic liver tumor, and cholangiocarcinoma, percutaneous needle biopsy was performed under CT because of the isoechoic lesion by ultrasonography. Histopathological examination showed marked infiltration of lymphocytes, plasma cells, fibrosis and loss of hepatic cells, findings consistent with IPT (Figure 2). Immunohistochemical staining for kappa- and lambda-light chains of immunoglobulin demonstrated the plasma cells contained both chains almost equally. The patient was followed up conservatively, and a repeat CT scan 3 mo after biopsy showed complete regression of IPT (Figure 1D). At this point, laboratory data (Table 1) showed normalization of gamma-GTP level and improvement of AMA (173.2 arbitrary unit) and IgM (281 mg/dL) levels except the same level of ANA (×160).

DISCUSSION

IPT is acute benign lesion that develops throughout the body. Hepatic IPT is relatively unique but has been recognized with increased frequency. It is difficult to make a specific diagnosis based on the findings of laboratory or imaging techniques because there is no specific laboratory marker and radiographic appearance. Therefore the vast majority of reported cases of IPT of the liver have been diagnosed after surgery or at autopsy[8-11]. Recently ultrasonography-guided percutaneus liver biopsy is reported to be useful[10,12].

The CT or MRI appearance of abdominal IPT are variable[13]. Moreover it has been reported that there are no specific signs of the disorder, in spite of the advances in imaging techniques (ultrasonography, endoscopic

retrograde cholangiography and angiography)[14-16], Yoon *et al*[17] reported that the area of low attenuation indicated the presence of chronic inflammatory infiltrations with foamy histiocytes, plasmacytes, and lymphocytes, while areas of iso- or high attenuation representes fibroblastic proliferation. Therefore the low attenuation of IPT on CT findings in our case may indicate a lower component of fibrosis and a higher component of cellular infiltration. Interestingly, to our knowledge, there is only one case of IPT detected by FDG PET, resulting from foreign body in abdominal cavity[18]. The tumor mass was identified as an inflammatory granuloma[18]. FDG PET may reflect inflammatory cell infiltrations in IPT in our case. The protean radiologic manifestations of IPT in our case may be the result not only of the variable morphologic structure including hyalinized collagen bundles or inflammatory cell infiltrations but the dynamic, rapidly changing nature of an inflammatory process.

While the exact etiology of hepatic IPT remains unclear, several mechanisms have been postulated such as infection, immune reaction, intraparenchymal hemorrhage and necrosis, occlusive phlebitis of intrahepatic veins, and secondary reaction to intrahepatic rupture of a biliary radical[19]. Cholangitis arising from various causes including infection with microorganisms, immunological or allergic reactions, and primary sclerosing cholangitis (PSC) has been also considered responsible for the development of hepatic IPT[1-4]. In only two recent reports[5,6], PBC was proposed as a possible cause of hepatic IPT. In both of these cases, hepatic IPT was detected with the simultaneous discovery of PBC or untreated PBC, associated with extremely high levels of biliary enzymes and autoimmune antibodies. Hyper gamma-globulinemia has been also suggested to contribute to the development of IPT[20-22]. The activity of cholangitis or hyper gamma-globulinemia as the etiological factor is, however, cannot be applied to our case because ANA (X5120), ALP (682 IU/L), gamma-GTP (248 IU/L), IgG (2010 mg/dL) and IgM levels (1010 mg/dL), measured when hepatic IPT had not been detected by CT before treatment of UDCA, were much higher than the corresponding laboratory data on admission (Table 1). At the time of regression of IPT, some of the laboratory data (Table 1) improved compared to those on admission probably due to effectiveness of UDCA add-on bezafibrate. However causal association between development or regression of IPT and improvement of laboratory data remains uncertain. Importantly, our case suggests IPT can develop at relatively inactive or recovery phase of PBC differing from previous reports[5,6] and on the contrary, IPT with PBC is probably not the same disease entity or a bystander for PBC.

The other finding exemplifying disease diversity is that the rapid resolution of some cases[4], including our case, may be related to the effectiveness of treatment of the underlying disease whereas in the other reported case[5] IPT regressed even though UDCA did not effectively improve the laboratory data. With regard to treatment of IPT, most reported patients underwent surgical resection because it is difficult to distinguish IPT from malignant liver tumors using only imaging modalities[9,23]. However, the prognosis of hepatic IPT is usually benign. Based on its spontaneous regression property, it is suggested that treatment of IPT should be conservative or noninvasive in patients diagnosed by imaging or needle biopsy[24]. As a treatment protocol, we recommend the use of medications first, such as UDCA and/or bezafibrate for patients with IPT and PBC or PSC after making a definite diagnosis. Once PBC is diagnosed, it is advised to follow such patients regularly and carefully by imaging studies to rule out the development of hepatic IPT during the clinical course, like our case. Since IPT rarely arises in PBC, but hepatocellular carcinoma often develops in cirrhotic patients with PBC, IPT may be one of hepatic tumors arising in PBC patients as a differential diagnosis during the clinical course. The coexistence of hepatic IPT and PBC, however, may be an accidental event. Further cases need to be diagnosed and studied in more detail.

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CASE REPORT

Malignant phyllode tumor metastatic to the duodenum

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Abstract

Phyllode tumor (PT) is extremely rare tumor of the breast. Distant metastasis occurs in 10-20% of patients with malignant phyllode tumor. The most common sites of metastases are the lungs and bones. Although theoretically any organ may have metastasis, an isolated duodenum metastasis has not been documented as yet in the English-language literature. We report herein a case with a isolated duodenal metastasis from PT of breast in a 31 year-old-woman who underwent right mastectomy 4 years before because of the recurrent malignant PT. She presented to our hospital with massive upper gastrointestinal bleeding. Clinical evaluation revealed a huge mass originated from duodenum. Urgent laparotomy and pancreaticoduodenectomy were carried out in order to remove the bleeding duodenal mass. The pathologic examination of the resected specimen showed a malignant spindle cell tumor consistent with metastatic malignant PT. Our case of gastrointestinal bleeding due to an isolated duodenal metastasis as a result of hematogenous spread from malignant phyllode tumor of breast is unique in the English literature and pancreaticoduodenectomy is a curative treatment for patients with isolated duodenal involvement.

Key words: Malignant phyllode tumor; Duodenal metastasis; Pancreaticoduodenectomy

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INTRODUCTION

Duodenal metastases of malignant tumors are rare. Lung cancer, renal cell carcinoma, breast carcinoma, and malignant melanoma are the most common primary tumors that metastasize to the pancreaticoduodenal region[1]. The proportion of duodenal metastases from breast carcinoma has been reported in autopsy series as occuring in more than 15% patients, generally associated with extensive systemic spread, and clinical presentations from such metastases have been described in less than 1% of cases[2]. Lobular infiltrating carcinoma of the breast is the histological type that most frequently metastasizes to the duodenum[2]. Infiltrating ductal carcinoma of the breast has also been reported to metastasize to the duodenum[2,3]. However, gastrointestinal metastases from phyllode tumor of breast have rarely been reported in the literature[3,4]. To our knowledge, this report is the first case in the English literature of isolated duodenal metastases of phyllode tumor.

CASE REPORT

A 31-year-old female presented to the surgical clinic with severe upper gastrointestinal bleeding (hematemesis and melena). She was diagnosed of right breast phyllode tumor for which an excision of 4 cm mass was performed 6 years ago. Two years later she had a recurrence, and the excisional biopsy revealed malignant phyllode tumor, and hence she underwent a right mastectomy. She refused postoperative radiation therapy for malignant PT.

At the time she presented to our hospital, she was hypotensive with a blood pressure of 80/60 mm/Hg, and had temperature 36.2℃ (97.1°F), pulse 106 bpm, and respiratory rate 18 breaths/min. She appeared pale, weak, and in distress, with a dry oral mucosa. A huge firm mass was palpable on epigastrium and upper right side on physical examination. Results of laboratory testing on admission showed 8g/dL hemoglobin, 23.6% hematocrit, 38 mg/dL blood urea nitrogen, and 1.8 g/dL albumin level. The results of coagulation tests (prothrombin time and partial thromboplastin time) were within normal limits.

Upper gastrointestinal endoscopic examination revealed clotted blood in the stomach and an ulcerated duodenal tumor without active bleeding. Computed tomography scan showed that the huge mass (10 cm ×15 cm in dimension) originated from duodenum, and compressedthe liver, pancreas, kidney, inferior vena cava, and superior mesenteric vein (Figure 1). Throughout testing, the patient remained hemodynamically unstable. A central line was placed, and

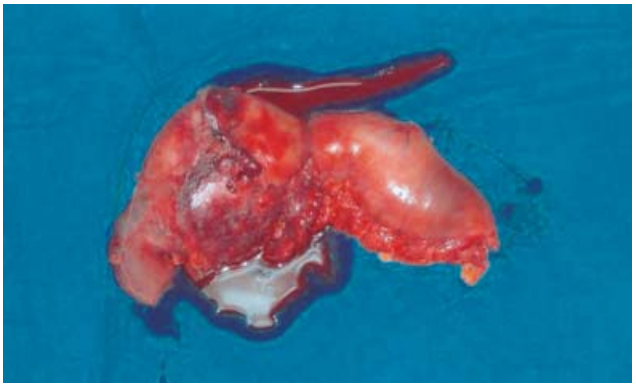

Figure 1 Specimen of the pancreaticoduodenectomy with negative margin.

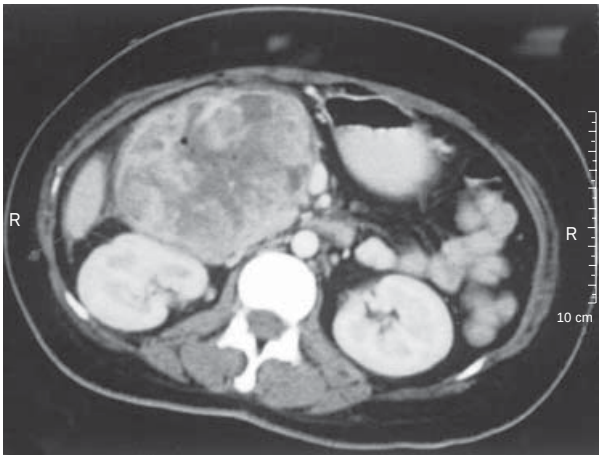

Figure 2 Computed tomography showing a 10 cm x 15 cm oval, mildly hypoechoic tumor with smooth margins.

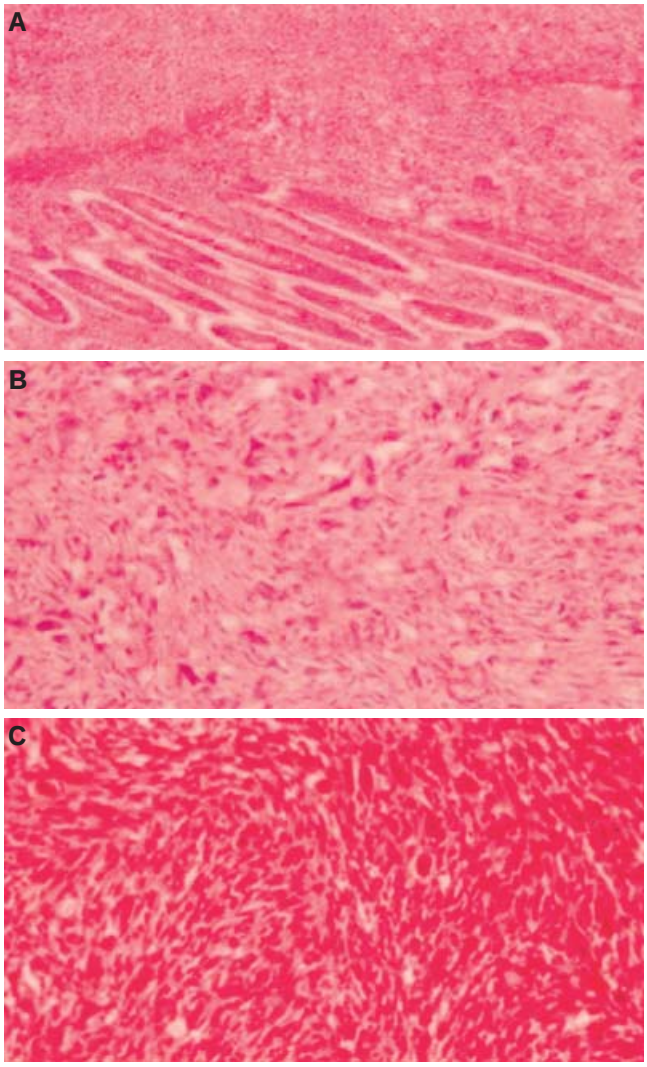

Figure 3 Neoplastic cells showing invasion of duodenal mucosa and submucosa (**A**) (H&E x 100); duodenal tumor consisted of haphazardly arranged pleomorphic spindle cells (**B**) (H&E x 200); breast tumor showing the similar histopathological findings with the duodenal tumor (**C**) (H&E x 200).

she received 8 units of packed erythrocytes. Since it was impossible to stop bleeding by embolisation of the huge mass, an urgent laparotomy and standard pancreaticoduodenectomy were carried out with the suspicion of primary duodenal tumor.

Gross examination of the resected specimen revealed a highly necrotic mass 15 cm×10 cm×10 cm in size within the wall of duodenum and pancreas with tumor-free resection margins (Figure 2). Histopathologically, tumor consisted of spindle cells with prominent nuclear pleomorphism and high mitotic index. Neoplastic cells showed invasion of all layers of duodenum, including mucosa (Figures 3A and 3B). Patient's mastectomy slides were obtained and compared to the duodenal tumor. Both tumors had the similar histopathological findings; atypical spindle cells showing an irregular cellular pattern without having an epithelial component (Figure 3C). Immunohistochemical analysis revealed only vimentin positivity in both tumors. The expressions of pan-cytokeratin, CD-117, smooth muscle actin, S-100, desmin and CD-34, CD-31 and CD-68 were all negative. Thus, a diagnosis of malignant spindle cell tumor consistent with metastatic malignant phyllode tumor was made.

The patient recovered uneventfully, and was discharged on the 10th postoperative day. The patient was alive and in good health 12 mo after surgery, with no evidence of disease at the moment.

DISCUSSION

Phyllode tumor is an uncommon fibroepithelial breast neoplasm that accounts for 0.5-1.0% of female breast tumor cases[5,6]. Phyllode tumors are generally classified into benign, borderline, and malignant tumors[5]. Malignant forms comprise nearly 25% of cases[7]. The rates of local recurrence and distant metastasis in patients with malignant PT range between 20-32% and 25-40%, respectively[5,6]. Positive or close surgical margin and large tumor size are important factors responsible for developing local recurrence[5,8]. They usually metastasize to the lung, pleura, bone, and liver but discrete localizations of metastases such as heart and central nervous system have also been reported[9,11]. The most reliable predictors for development of distant metastasis are stromal overgrowth, nuclear pleomorphism and high mitotic activity[6,7]. Mixed mesen-

chymal components (osteosarcomatosis, chondrosarcom *etc*.) have also been reported to be associated with worse prognosis[7]. Whether both local recurrence and tumor size contribute to the development of distant metastasis is still controversial[5,7,8]. In our case, the tumor size was 4 cm and the patient had a local recurrence 4 years ago.

Distant metastases of PT usually develop within 3 years of initial therapy, they may occur as soon as synchronous presentation or as long as more than ten years[7]. Because lungs are the most common site of metastasis for malignant phyllode tumors, all patients diagnosed with either a borderline or malignant lesion should undergo CT scanning of the chest prior to definitive surgery. This scan should include the liver, although liver metastases are less common. The primary goal for treatment of localized phyllode tumor is to achive wide, negative margin excision, this is generally agreed to include margins of 1 cm or greater.

We would like to stress that the preoperative diagnosis of a secondary gastrointestinal tumor begins with suspicion based on the history of a previously known non-gastrointestinal tumor[12,13]. In the presence of gastrointestinal mass in patients with a history of previous malignant PT, the physician should be aware of the possibility of gastrointestinal metastasis. The second primary tumor is known to be more common than isolated gastrointestinal metastasis. It is crucial to distinguish a metastatic gastrointestinal tumor from a primary one because of the therapeutic and prognostic implications[1,13]. Gastrointestinal metastasis can occur anywhere in the gut and can cause gastrointestinal symptoms, such as intestinal obstruction or bleeding. Radiological and endoscopic evaluations are usually nonspecific. Moreover, even histopathologically it is sometimes very difficult to distinguish a primary gastrointestinal stromal sarcoma or other sarcomas from a metastatic PT. In our case, we applied immunohistochemically a panel of antibodies to both duodenal and breast tumors. Results were not in concordance with other primary tumors. The histopathological findings were very similar to malignant PT, although we could not find any epithelial component, but it is a very well phenomenon that the metastases are commonly of stromal elements only in malignant PT[5].

To our best of knowledge, this is the first report of a PT metastasis to the duodenum. Currently, there are no clinical trials available to assess the best treatment regimen for metastatic PT. Experience with systemic therapy for metastatic PT is anecdotal. Chemotherapy may occasionally afford some palliation[14,15]. Ifosfamide is thought to be the most active agent. There is no demonstrated role for hormonal therapy. Given this limited experience, adjuvant systemic therapy cannot be recommended even for poor prognosis tumors. However, surgical treatment may become an ultimate option for patients who develop complication such as bleeding from the metastatic tumor, as seen in our case. Because of the therapeutic and prognostic implications, it is important to distinguish a metastatic gastrointestinal tumor from a primary one.

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World J Gastroenterol 2006 March 14; 12(10): 1653
World Journal of Gastroenterology ISSN 1007-9327

Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
Falk Foundation e.V.
symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

14th United European Gastroenterology Week
21-25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25-28 October 2006
Berlin
comtec international
codhy@codhy.com
www.codhy.com

Asia Pacific Obesity Conclave
1-5 March 2006
New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11-16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
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6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

Hepatitis 2006
25 February 2006-5 March 2006
Dakar
hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24-26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20-23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26-28 January 2006
San Francisco - CA
Gastrointestinal Cancers Symposium
Registration Center
giregistration@jspargo.com

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
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American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

71st ACG Annual Scientific and Postgraduate Course
20-25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
www.nysge.org

EVENTS AND MEETINGS IN 2007

9th World Congress on Gastrointestinal Cancer
20-23 June 2007
Barcelona
Imedex
meetings@imedex.com

Gastro 2009, World Congress of Gastroenterology and Endoscopy London, United Kingdom 2009

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An informative, structured abstract of no more than 250 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipments, and the experimental procedures should be included. RESULTS: The observatory and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and the significant data should accompany. CONCLUSION: Accurate view and the value of the results should be included.

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Data that are not statistically significant should not be noted. $^{a}P<0.05$, $^{b}P<0.01$ should be noted ($P>0.05$ should not be noted). If there are other series of *P* values, $^{c}P<0.05$ and $^{d}P<0.01$ are used. Third series of *P* values can be expressed as $^{e}P<0.05$ and $^{f}P<0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or some other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.* in a certain sequence.

Acknowledgments
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1 **Das KM,** Farag SA. Current medical therapy of inflammatory bowel disease. *World J Gastroenterol* 2000; 6: 483-489 [PMID: 11819634]

2 **Pan BR**, Hodgson HJF, Kalsi J. Hyperglobulinemia in chronic liver disease: Relationships between *in vitro* immunoglobulin synthesis, short lived suppressor cell activity and serum immunoglobulin levels. *Clin Exp Immunol* 1984; 55: 546-551 [PMID: 6231144]

3 **Lin GZ,** Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; 7: 285-287

Books and other monographs (list all authors)

4 **Sherlock S,** Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

5 **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

Electronic journal (list all authors)

6 **Morse SS.** Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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Statistical data

Present as mean ± SD and 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 γ (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.

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Italicis

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

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World J Gastroenterol 2006 March 14; 12(10): 1656
World Journal of Gastroenterology ISSN 1007-9327

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Number	Nonstandard	Standard	Notice
1	4 days	4 d	In figures, tables and numerical narration
2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
5	2 hours	2 h	After Arabic numerals
6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0,1,2 yr	In figures and tables
12	0,1,2 year	0,1,2 yr	In figures and tables
13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
15	2 months	2 mo	In figures and tables
16	Two mo	Two months	At the beginning of a sentence
17	10 minutes	10 min	
18	Ten min	Ten minutes	At the beginning of a sentence
19	50% (*V/V*)	500 mL/L	
20	50% (m/*V*)	500 g/L	
21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_4	
25	4rd edition	4^{th} edition	
26	15 year experience	15- year experience	
27	18.5 kDa	18.5 ku,18 500u or M_r18 500	
28	25 $g.kg^{-1}/d^{-1}$	25 g/(kg·d) or 25 g/kg per day	
29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	13 000 rpm	13 000 *g*	High speed; *g* should be in italic and suitable conversion.
35	1000 *g*	1 000 r/min	Low speed. *g* cannot be used.
36	Gene bank	GenBank	International classified genetic materials collection bank
37	Ten L	Ten liters	At the beginning of a sentence
38	Ten mL	Ten milliliters	At the beginning of a sentence
39	umol	μmol	
40	30 sec	30 s	
41	1 g/dl	10 g/L	10-fold conversion
42	OD_{260}	A_{260}	"OD" has been abandoned.
43	One g/L	One microgram per liter	At the beginning of a sentence
44	*A*260 nm	A_{260} nm	*A* should be in italic.
	$^bP<0.05$	$^aP<0.05$	In Table, no note is needed if there is no significance instatistics: $^aP<0.05$, $^bP<0.01$ (no note if $P>0.05$). If ther is a second set of *P* value in the same table, $^cP<0.05$ and $^dP<0.01$ are used for a third set: $^eP<0.05$, $^fP<0.01$.
45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=67.4	Notices in or under a table
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	*x*±s	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
70	*iv*	iv	intravenous injection
71	Wang et al	Wang *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	