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Contents

Contents

World Journal of Gastroenterology
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Volume 13 **Number** 42 **November** 14, 2007

Contents

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EDITORIAL

Emerging role of IL-23/IL-17 axis in *H pylori*-associated pathology

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Abstract

Colonization of stomach by *H pylori* is followed by a marked infiltration of the mucosa with polymorphonuclear leukocytes, macrophages, and lymphocytes that very often remains asymptomatic, but in some circumstances can lead to the development of gastroduodenal ulceration, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma. The molecular mechanisms by which *H pylori* triggers and maintains the local immune response are complex, but there is evidence that cytokines produced by both immune and non-immune cells contribute to amplify the ongoing inflammation. *H pylori* infection is associated with a marked mucosal induction of T helper (Th) type 1 and Th17-type cytokines that is governed by specific antigen-presenting cell-derived molecules, such as interleukin (IL)-12 and IL-23. In this paper, we will review the available data on the expression and role of IL-23 and IL-17 in *H pylori*-related gastritis.

Key words: IL-23; IL-17; *H pylori*

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INTRODUCTION

H pylori is a spiral-shaped Gram-negative flagellate bacterium that colonizes the human gastric mucosa and chronically infects more than half of the human population. Infection is inversely correlated with socioeconomic conditions. Most new *H pylori* infections occur in children, but the lack of specific *H pylori*-related clinical signs makes difficult to define the mode of transmission[1]. *H pylori* survives within the gastric mucus layer despite the acidic microenvironment, that limits the growth of most bacteria. This primarily relies upon the ability of *H pylori* to secrete a large amount of urease that breaks down urea into carbon dioxide and ammonia, the latter buffering its environment. Most *H pylori* organisms remain in the mucus layer, even though a small proportion adheres to the mucosal epithelial cells and rarely invades the mucosa[2]. Moreover, *H pylori* can inject into the epithelial cells bacterial products that modify epithelial cell functions[3].

IL-17 IS OVER-PRODUCED IN *H pylori*-COLONIZED GASTRIC MUCOSA

H pylori infection causes a marked infiltration of the gastric mucosa with neutrophils, macrophages, and lymphocytes. Most *H pylori*-infected patients are asymptomatic, but *H pylori*-driven gastritis can lead to the development of gastroduodenal ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma[4]. The level of inflammation increases the risk of disease, but it does not seem to influence which disease develops. In contrast, this is thought to be largely influenced by the pattern of gastric inflammation. In particular, antral gastritis is associated with increased stimulated acid production and predisposes to duodenal ulceration, while corpus-predominant or pangastritis is associated with reduced acid production and predisposes to gastric ulcer and gastric adenocarcinoma[5]. There is also evidence that the degree of gastric infiltration by neutrophils correlates with the development of gastroduodenal ulcerations, and this is in part dependent on the release of damaging inflammatory mediators such as reactive oxygen species[6,7]. Because neutrophils are short-lived, they must be constantly recruited into the infected mucosa from circulation. Antigens released by *H pylori* can stimulate endothelial cells, macrophages and epithelial cells to make huge amounts of chemokines, such as interleukin (IL)-8 and growth-regulated oncogene-alpha, that produce a chemotactic gradient for the migration of neutrophils into the gastric mucosa[8-11]. It is also known that infections with specific *H pylori* strains that possess the cag pathogenicity island (*cag*+) induce significantly higher levels of chemokines than do cag-strains[12]. Both macrophages and epithelial cells also synthesize neutrophil-recruiting chemokines in response to lamina propria mononuclear cell

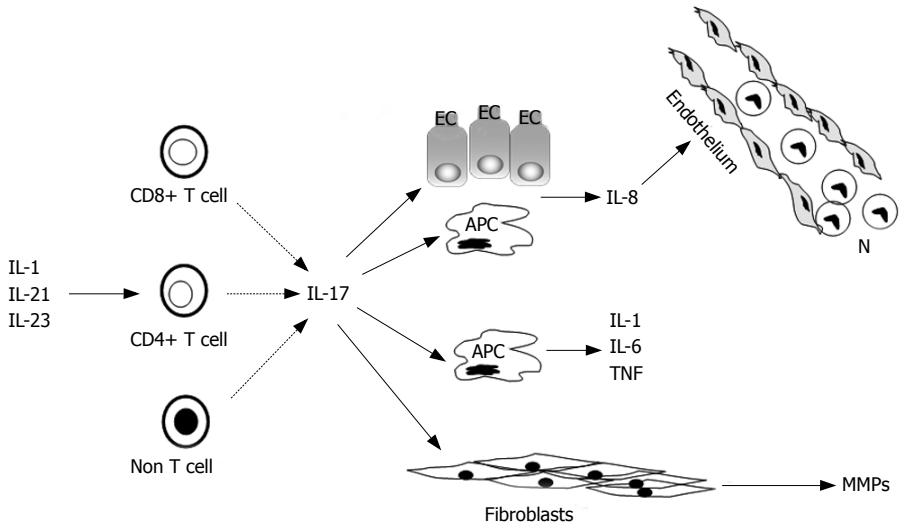

Figure 1 The figure illustrates some of the putative functions of IL-17 in the human gastric mucosa. During *H pylori* infection, IL-17 is produced by both lamina propria (LP) T (CD4+ and CD8+) and non-T cells, through a process that could be positively regulated by IL-1, IL-21, and IL-23. IL-17 stimulates both epithelial cells (EC) and LP antigen presenting cells (APC) to make IL-8, thereby enhancing the recruitment of blood neutrophils (N) into the mucosa. Additionally, IL-17 increases the production of inflammatory cytokines, such as IL-1, IL-6, and TNF by LP APC, as well as it stimulates fibroblasts to secrete matrix metalloproteinases (MMPs), a family of proteases that can cause mucosal degradation.

(LPMC)-derived molecules. In this context, we and others have recently shown that IL-17, a key regulator of neutrophil chemotaxis, is produced in excess in *H pylori*-infected stomach[13-15]. By real-time PCR and Western blotting it was shown that IL-17 up-regulation occurs at both RNA and protein levels in *H pylori*-infected biopsies in comparison to uninfected biopsies either with or without gastritis[13,14]. Notably, among *H pylori*-positive patients, the gastric mucosa at the site of ulcers contains more IL-17 than the non-ulcerated mucosa of the antrum[15]. Several observations suggest that IL-17 plays a decisive role in the neutrophil recruitment to the *H pylori*-infected gastric mucosa. First, IL-17 levels correlate with the number of neutrophils infiltrating the Hp-infected mucosa[15]. Second, both gastric LPMC and epithelial cells express IL-17 receptors and are functionally capable of responding to IL-17 by secreting IL-8[14-16]. Consistently, conditioned media of gastric epithelial cells stimulated with IL-17 enhance the migration of peripheral blood neutrophils, and this effect is inhibitable by a blocking anti-IL-8, but not anti-IL-17 antibody (Figure 1)[14]. Functional analysis of intracellular pathways involved in the induction of IL-8 synthesis by IL-17 revealed that IL-17 activates ERK1/2 MAP kinases in gastric epithelial cells, and that pharmacologic blockade of this pathway significantly inhibits IL-8 secretion[16]. These findings are in line with the demonstration that activated ERK1/2 and IL-8 are more pronounced in gastric epithelial cells isolated from *H pylori*-infected biopsies in comparison to uninfected controls, and that neutralization of endogenous IL-17 in *ex vivo* cultures of *H pylori*-infected gastric biopsies down-regulates the expression of activated ERK1/2 and IL-8[16]. Finally, IL-17 expression positively correlates with IL-8 content in *H pylori*-colonized biopsies[15].

Besides its effects on IL-8 synthesis, IL-17 exerts additional immune-regulatory functions which could influence the magnitude and/or severity of *H pylori*-related gastritis. For example, IL-17 stimulates the production of IL-1, IL-6, and TNF-α by both immune and non-immune cells[17], and induces fibroblasts to make matrix metalloproteinases (MMPs)[18]. MMPs are a family of proteases that can cleave multiple components of the extracellular matrix, thereby contributing to the mucosal damage[19] (Figure 1).

IL-23 CONTROLS IL-17 PRODUCTION IN THE HUMAN GASTRIC MUCOSA

IL-17 was originally named cytotoxic T lymphocyte-associated-8 (CTLA-8), subsequently IL-17, and more recently IL-17A, since it is one of six related members belonging to the IL-17 family (IL-17A-F)[20]. IL-17 was initially described at the message level as a product of human blood activated CD4+ memory T cells. Subsequent studies have shown that IL-17 can be also made by activated CD8+ T cells, TCR$\gamma\delta$+ T cells, and neutrophils[20]. More recently, it was shown that IL-17 is produced by a specific subset of CD4+ T cells, termed T helper (Th) 17-cells, that are distinct from, and antagonized by the classical Th1 or Th2 cells[21]. Th17-cells produce also, but to a lesser extent, TNF-α, IL-6, IL-17F, IL-22, and granulocyte macrophage-colony stimulating factor[22,23]. Flow-cytometry analysis of IL-17 production in gastric LPMC isolated from biopsies of *H pylori*-infected patients showed that CD4+ T cells are a major source of IL-17, even though CD8+ T cells and CD3-negative cells were also positive for IL-17 (Figure 1)[13]. The molecular pathways governing the development of Th17-cells in humans have not been yet elucidated, but studies in murine systems indicate that Th-17 cell differentiation is driven by IL-6 and TGF-β1[24,25]. There is also evidence that expansion and survival of Th17-cells require additional factors, such as IL-23[25]. IL-23 is a heterodimeric protein that is composed by the p40 subunit of IL-12 and a specific subunit, termed IL-23/p19. The functional IL-23 heterodimer is produced by activated dendritic cells (DC), monocytes and macrophages[26]. We have recently shown that IL-23 protein is produced in excess in *H pylori*-colonized mucosa. RNA transcripts for both p40 and p19 subunits were also up-regulated in biopsies from *H pylori*-infected patients, indicating that IL-23 is regulated at the transcriptional level in this condition[13]. These results confirm and expand on data of previous studies showing that *H pylori* enhances IL-23 secretion by monocyte-derived DC[27], and that *H pylori* neutrophil-activating protein (*H pylori*-NAP), a member of a broad super-family of ferritin-like proteins, induces IL-23 production by neutrophils and monocytes[28,29]. Functional studies also revealed that

IL-23 enhances IL-17 synthesis by normal gastric LPMC, and that blockade of endogenous IL-23 activity in cultures of LPMC isolated from *H pylori*-infected biopsies down-regulates IL-17 production[13]. The exact molecular mechanism by which IL-23 regulates IL-17 in *H pylori*-infected mucosa remains to be ascertained. Notably, neutralization of endogenous IL-23 by a blocking anti-IL-23/p19 antibody in cultures of LPMC isolated from *H pylori*-infected biopsies attenuates the expression of active Stat3. Moreover, in normal gastric LPMC, exogenous IL-23 enhances the activation of Stat3, and pharmacologic inhibition of Stat3 suppresses IL-17 production induced by IL-23[13]. Taken together, these results suggest that Stat3 plays a key role in the IL-23-driven IL-17 production during *H pylori* infection. This well fits with the demonstration that Stat3 is essential for the induction and expansion of IL-17-producing cells in response to cytokine stimulation both in vitro and *in vivo*[30]. Such an effect could rely on the ability of Stat3 to bind the promoter of IL-17 gene and enhance its transcriptional activity[31], and/or favor the induction of RORγt, a master regulator of Th17-cell differentiation[32], and the expression of IL-23R.

IL-17 synthesis may be regulated by additional cytokines other than IL-23. IL-1R1-deficient mice fail to mount a robust Th17 response, and IL-1R1-deficient cells do not produce IL-17 in response to IL-23[33]. Since *H pylori* infection enhances the production of IL-1 at the gastric level[34], it is tempting to speculate that this cytokine may act in concert with IL-23 in enhancing IL-17. IL-17 synthesis is also increased by IL-15 in cultures of human and murine CD4+ T cells[35]. However, the fact that IL-15 expression is down-regulated in *H pylori*-infected biopsies argues against a role for IL-15 in the control of IL-17 production during *H pylori*-related gastritis[36]. Th17 cell differentiation is also enhanced by IL-21[37,38], a T-cell derived cytokine that is produced in excess in *H pylori*-colonized stomach[39].

THE ROLE OF IL-23 IN AMPLIFYING *H pylori*-DRIVEN TH1-IMMUNE RESPONSE

During *H pylori* infection, there is a pronounced specific acquired immune response, characterized by generation of antibodies, and differentiation and activation of effector T cells. Although this later includes both a Th1 and a Th2 component, mucosal cytokine profiles imply Th1 predominance[40], and the number of cells producing interferon (IFN)-γ, the key Th1 cytokine, in the *H pylori*-infected human gastric mucosa correlates with the severity of gastritis[41]. Animal models also suggest that the extent of Th1 differentiation is important in pathogenesis. Mice with a predominant Th1 response develop more gastric inflammation during *H pylori* colonization than those with a Th2 response[42-43]. Gastric inflammation and atrophic changes are abrogated in the absence of IFN-γ[44], while IFN-γ infusion into mice, even in the absence of *H pylori* infection, induces pre-cancerous gastric atrophy, metaplasia and dysplasia[45]. IL-12-deficient mice have also reduced gastric inflammatory infiltration and are unable to clear *H pylori* infection[46].

Several virulence factors are reported to promote Th1 responses, including the plasticity region locus jhp0947-jhp0949 which is associated with duodenal ulcer disease[47] and the *H pylori*-NAP[29]. The Th1/Th2 balance is also influenced by phase-variable expression of Lewis blood-group antigens and genomic DNA recombination[48,49].

IL-12 is supposed to be one of the major Th1-inducing factors in *H pylori*-colonized gastric mucosa[50], even though IL-23 may contribute to expand the ongoing Th1 cell response[26]. Indeed, blockade of endogenous IL-23 by anti-IL23/p19 in cultures of LPMC isolated from *H pylori*-colonized biopsies reduces IFN-γ secretion, and stimulation of normal gastric LPMC with IL-23 enhances IFN-γ production. These data are in accordance with the demonstration that IL-23 activates Stat4 and enhances IFN-γ production in cultures of human and murine memory T cells[26], and that in two models of *H. hepaticus*-triggered T cell-dependent colitis, IL-23 enhances both IFN-γ and IL-17 responses that together synergize to trigger severe intestinal inflammation[51,52].

IL-23 and gastric cancer

As pointed out above, *H pylori* is a major factor in the induction of gastritis and its progression to pre-neoplastic lesions and non-cardia gastric cancer. Despite the high prevalence of *H pylori* infection, the risk of gastric cancer in *H pylori*-infected patients is, however, estimated to be approximately 1%-3%. This indicates that infection per se is not sufficient to induce the progression to gastric neoplasia and that additional bacterial and host factors are required[2]. A detailed description of such factors is beyond the scope of this review. In this context it is, however, noteworthy that accumulating evidence would seem to suggest that IL-12 and IL-23 are important mediators in the process that links *H pylori* infection to gastric cancer. Indeed, polymorphisms of IL-12p40 and p35 genes enhance the risk of non-cardia gastric cancers in *H pylori*-infected patients[53]. Moreover, high levels of IL-23 have been documented in human gastric cancers[53]. Nonetheless, no functional study has so far mechanistically linked the activity of IL-12 and IL-23 to gastric cancer. Additionally, studies in murine models of epithelial cancers have shown that IL-23, but not IL-12, is essential for sustaining the tumor-promoting inflammatory process and counteracting the ability of cytotoxic CD8+ T cells to infiltrate tumors[54].

CONCLUSIONS

Although bacterial virulence factors are important in conditioning the outcome of the *H pylori*-driven infection, it is the host attempt to clear the bacterium that causes an exaggerated and inappropriately counter-regulated immune response that may eventually cause tissue damage. Emerging experimental evidence suggests that IL-23/IL-17 pathway is an important driving force the ongoing gastric inflammation in *H pylori*-infected patients. However, further studies will be required to establish the exact contribution of each of these cytokines in the *H pylori*-associated gastric pathology. The availability of strains of mice deficient either for IL-23 subunits or IL-17 should provide valuable models to specifically address these issues.

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REVIEW

Acute renal dysfunction in liver diseases

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Abstract

Renal dysfunction is common in liver diseases, either as part of multiorgan involvement in acute illness or secondary to advanced liver disease. The presence of renal impairment in both groups is a poor prognostic indicator. Renal failure is often multifactorial and can present as pre-renal or intrinsic renal dysfunction. Obstructive or post renal dysfunction only rarely complicates liver disease. Hepatorenal syndrome (HRS) is a unique form of renal failure associated with advanced liver disease or cirrhosis, and is characterized by functional renal impairment without significant changes in renal histology. Irrespective of the type of renal failure, renal hypoperfusion is the central pathogenetic mechanism, due either to reduced perfusion pressure or increased renal vascular resistance. Volume expansion, avoidance of precipitating factors and treatment of underlying liver disease constitute the mainstay of therapy to prevent and reverse renal impairment. Splanchnic vasoconstrictor agents, such as terlipressin, along with volume expansion, and early placement of transjugular intrahepatic portosystemic shunt (TIPS) may be effective in improving renal function in HRS. Continuous renal replacement therapy (CRRT) and molecular absorbent recirculating system (MARS) in selected patients may be life saving while awaiting liver transplantation.

Key words: Hepatorenal syndrome; Transjugular intrahepatic portosystemic shunt; Continuous renal replacement therapy; Molecular absorbent recirculating system; Acute liver failure; Systemic vascular resistance; Renal blood flow

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INTRODUCTION

Renal and liver dysfunction often present together, either as part of multiorgan failure in a critically ill patient, or as a result of failure of each organ independently. Three major clinical scenarios can be identified in which liver and renal dysfunction coexist; diseases simultaneously involving the liver and the kidney, or a primary hepatic disorder with secondary renal dysfunction, or vice versa[1]. Concomitant renal and liver dysfunction may share common pathogenetic mechanisms. Renal dysfunction in this setting usually develops gradually, with the exception of certain infections such as leptospirosis, some viral hemorrhagic fevers and toxin-mediated injuries such as acetaminophen poisoning, which cause acute insufficiency of both organs[2]. Renal failure secondary to liver dysfunction is generally functional in nature and occurs in the absence of significant alterations in renal histology (pre-renal). However, intrinsic renal abnormalities can also complicate acute or chronic liver disease (intrinsic renal failure)[3]. Obstructive uropathy that leads to postrenal acute renal failure only rarely develops in chronic liver disease (papillary necrosis in alcoholic liver disease, bleeding in the urinary tract due to severe coagulopathy)[4]. Hepatorenal syndrome (HRS) is a unique form of functional renal failure (pre-renal) that often complicates advanced liver disease, hepatic failure or portal hypertension[5].

EPIDEMIOLOGY

The incidence of renal failure in acute liver failure (ALF) varies from 40% to 85%, depending on the etiology; paracetamol poisoning leads to renal failure in up to 75% of patients[6]. Renal failure following paracetamol overdose may also occur in the absence of ALF, and has a good prognosis[7]. In non-paracetamol cases the incidence of renal failure is usually accompanied by worsening encephalopathy and is associated with a poor outcome[5,6].

Acute renal failure (ARF) in patients with cirrhosis, particularly with advanced liver disease, seems to be common; however, the exact incidence is unknown and is probably underestimated[3]. This may be explained by the fact that patients with cirrhosis tend to have falsely low serum creatinine levels due to decreased hepatic creatinine synthesis and decreased skeletal muscle mass[8]. ARF in patients with cirrhosis frequently accompanies complications such as bacterial peritonitis or other

sepsis, hypovolemia from gastrointestinal bleeding or excessive diuretic therapy, administration of nephrotoxic drugs/contrast agents, or development of HRS[2,3]. The probability of the occurrence of HRS in patients with cirrhosis and ascites at 1 and 5 years is 18% and 39%, respectively, with mortality approaching 100% in type I HRS without specific therapy. The median survival time in these patients without liver transplantation was only 12 d after diagnosis in one study[9]. However, this seems to have improved with terlipressin and albumin therapy[10]. The development of ARF in patients with cirrhosis has significant prognostic importance. In patients with cirrhosis admitted to hospital with acute upper gastrointestinal hemorrhage, development of ARF forms an independent predictive factor for death[11,12].

PATHOPHYSIOLOGY OF RENAL FAILURE IN LIVER DISEASE

The mechanism underlying the development of ARF in advanced liver disease and cirrhosis is complex and includes interactions between changes in the systemic arterial circulation, portal hypertension, activation of vasoconstrictors and suppression of vasodilatory factors acting on the renal circulation[1,3,13]. The pathophysiology of functional renal failure in ALF is similar to that in cirrhosis[6,14], and patients with ALF may develop portal hypertension, but to a lesser degree than in those with cirrhosis[6]. The common pathway of renal dysfunction is the development of intense systemic arterial vasodilation, which follows increased release of endogenous vasodilators, especially nitric oxide, which escapes from the splanchnic to the systemic circulation through portosystemic shunts[1,13-15]. The systemic vasodilation leads to a reduction in systemic vascular resistance (SVR) and consequent high cardiac output and hyperdynamic circulation. However, the increase in cardiac output may be inadequate to compensate for the drop in SVR, especially in ALF, which results in hypotension with mean arterial pressure (MAP) commonly falling to 60-70 mmHg, which is on the pressure-dependent part of the autoregulatory curve of renal blood flow (RBF)[6,16]. In healthy individuals, autoregulation of RBF occurs until the renal perfusion pressure falls below 60-70 mmHg. Altered renal vascular autoregulation, as seen in sepsis, may also be present in the hyperdynamic circulatory failure of ALF, making RBF directly dependent on blood pressure[17]. In some patients with cirrhosis, especially alcoholics, the presence of cardiomyopathy and heart failure may further render them susceptible to renal compromise secondary to hypoperfusion[18].

The normal homeostatic response to vasodilation is activation of several neurohumoral mechanisms, such as the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system (SNS), and arginine-vasopressin (AVP) which leads to intense vasoconstriction and salt and water retention, in an attempt to maintain blood pressure and perfusion of vital organs[2,5,14,17]. Other vasoconstrictors, such as eicosanoids, endothelins, thromboxane A2 and leukotrienes may further exacerbate this[6,13,14,19].

RBF is kept within normal limits in the early stage of the liver disease, due to the release of certain local vasodilators such as prostaglandins. However, in situations in which circulatory volume is acutely diminished, as in gastrointestinal hemorrhage, renal hypoperfusion and subsequent pre-renal azotemia may occur. As the liver disease progresses, there is extreme vasoconstriction of the renal vascular bed that predisposes the kidneys to development of HRS[2,20]. The presence of tense ascites may further impair renal perfusion. The continuing vasoconstriction and raised vascular resistance results in contraction of the mesangium, with a reduction in glomerular surface area, which leads to acute tubular necrosis (ATN)[21].

CAUSES OF ACUTE RENAL FAILURE IN LIVER DISEASE

Pre-renal

Patients with advanced liver disease are susceptible to pre-renal azotemia, secondary to the development of relative hypovolemia and reduced effective central blood volume. The initial event is development of portal hypertension, which then leads to splanchnic and systemic vasodilatation mediated by NO and other vasodilators. Vasodilatation seems to be the main mechanism, however, underfilling has also been suggested, which is explained by fluid sequestration in the peritoneal cavity[22-24]. True hypovolemia can further exacerbate renal dysfunction in these patients. It can be induced by gastrointestinal tract hemorrhage from varices, peptic ulcers, gastropathy or other sources, excessive diuresis, vomiting and diarrhea, or can be aggravated by large volume paracentesis without intravascular volume replacement[3,11,13]. Bacterial infections and the use of nonsteroidal anti-inflammatory drugs can also precipitate pre-renal azotemia in these patients[3,25]. Patients with ALF and cirrhosis are abnormally susceptible to infection. Hemodynamic abnormalities induced by cytokines and vasodilating substances, such as NO in spontaneous bacterial peritonitis, play an important role in the pathogenesis of renal dysfunction. Additionally, development of septic shock further impairs renal function[25,26].

Intrinsic renal

Intrinsic renal disease can either complicate acute liver diseases as a result of exposure to certain drugs, toxins and infections, or be a part of chronic liver disease. The former usually is tubular interstitial in nature and presents as ARF, while the latter predominantly leads to glomerulopathy and is characterized by stable kidney disease. The causes of intrinsic renal involvement in liver diseases are numerous and are beyond the scope of this review. This review will mainly concentrate on acute tubular necrosis (ATN) and HRS, with other causes listed in the Table 1.

ATN

Direct cellular toxicity with ATN and hepatocyte necrosis have been observed in paracetamol intoxication[16]. Despite the nephrotoxic potential of this drug, functional renal failure is also seen secondary to ALF. Renal failure rarely

occurs in the absence of liver failure[7]. Depletion of glutathione is believed to be the cause of both ARF and ALF[36]. Aspirin is another analgesic drug that can cause dose-related liver damage and renal failure in susceptible patients. Of special interest is the association of aspirin, used to treat symptoms of influenza or varicella, with Reye's syndrome[27,37]. The mechanism of renal damage is due to cyclooxygenase inhibition thereby preventing the production of vasodilatory prostaglandins.

ATN with acute renal insufficiency in patients with stable liver disease often follows insults such as hypovolemic shock, major surgical procedures and use of nephrotoxic drugs or contrast agents, infection or sepsis. The functional renal abnormalities associated with advanced liver disease and cirrhosis increase the susceptibility of the kidneys to the development of ATN. These renal abnormalities may either be ischemic or toxic in origin. The mechanism of renal failure is similar in both, and results from a reduction in glomerular filtration rate (GFR) due to impaired glomerular capillary pressure, disrupted integrity of tubular epithelium, and tubular obstruction from casts composed of detached epithelial cells, cellular debris and pigments (hemoglobin and myoglobin)[38]. It should be noted that all causes of pre-renal azotemia might lead to ischemic tubular injury if left untreated[21,39].

The association of obstructive jaundice and ARF is well-established. Susceptibility to renal failure in obstructive jaundice is a combination of cardiovascular instability due to defective vascular reactivity and blunted myocardial contractile response as a result of the deleterious effects of bile constituents. Furthermore, the natriuretic effects of bile acids can cause volume depletion and exaggerate the effective arterial underfilling. However, there is a direct nephrotoxicity of biliary products at very high levels of bilirubin (> 30 mg/dL) in both children and adults[40,41].

Contrast medium is a well-known precipitant of renal failure in hospitalized patients, particularly in the presence of predisposing conditions such as reduced effective blood volume, dehydration and diabetes mellitus. Cirrhosis has been considered a potential predisposing factor. However, a prospective study in euvolemic patients with cirrhosis has shown that administration of contrast medium is not associated with adverse effects on renal function, which suggests that cirrhosis per se should not be considered a risk factor for contrast media nephrotoxicity[42,43]. However, it may play a role in septic cirrhosis, or in patients who have bleeding or who have undergone transarterial embolization for hepatocellular carcinoma.

Table 1 Intrinsic kidney involvement in liver diseases

Tubulo-interstitial involvement
1 Drugs[7] (paracetamol, aspirin, carbon tetrachloride, halogenated hydrocarbons, immunosuppressant agents)
2 Toxins[28-35] (Galerina family of mushrooms, hemoglobin, myoglobin, bilirubin, contrast agents)
3 Infections (leptospirosis, malaria, hepatitis)
4 Hypersensitivity reactions (sulphonamides, salicylates, *etc.*)
Glomerular involvement
1 Drugs[7] (carbon tetrachloride)
2 Hepatitis[28-35] A, B, C
3 Type Ⅱ mixed cryoglobulinemia[28-35]
4 IgA nephropathy[28-35] (alcoholic cirrhosis, HCV cirrhosis)
5 Others (sickle cell disease, hemochromatosis, acute fatty liver and toxemia of pregnancy)
Vascular
1 Vasculitis
2 Toxemia of pregnancy and HELLP syndrome

HRS

HRS is a unique form of functional renal failure that often complicates advanced liver disease, hepatic failure or portal hypertension[3,9,20]. The incidence of HRS in patients with cirrhosis hospitalized for ascites is about 10%. The syndrome is characterized by intense intrarenal vasoconstriction in the presence of vasodilation of systemic and splanchnic circulation, which triggers a reduction in peripheral vascular resistance and a decrease in effective systemic circulatory volume, despite an overall expanded total extracellular fluid volume. The majority of patients have clinical evidence of advanced cirrhosis[20]. However, HRS may occur in patients with fulminant viral and alcoholic hepatitis[36]. Two patterns of HRS can be identified.

Type 1 HRS is characterized by a rapidly progressive reduction of renal function, defined as either doubling of the initial serum creatinine to > 2.5 mg/dL or a 50% reduction in GFR to < 20 mL/min over a 2-wk period. Precipitating factors include spontaneous bacterial peritonitis (SBP), major surgical procedures, and acute alcoholic hepatitis. It follows a fulminant course with development of oliguria, encephalopathy, and marked hyperbilirubinemia, and is associated with very poor prognosis, with death occurring within 1 mo after presentation[27,36].

Type 2 HRS is characterized by a more benign course, with a stable reduction in GFR over weeks to months, accompanying diuretic-resistant ascites and avid sodium retention[36]. The pathogenesis of HRS is incompletely understood. It may be the result of an imbalance between renal vasodilators and vasoconstrictors, with the latter predominating. This interplay between the intrarenal mechanisms is triggered by one of the above-mentioned precipitating factors, which exacerbate the previously diminished cardiac and renal function[14,20].

The diagnostic criteria of HRS as proposed by the International Ascites Club are listed in Table 2[44]. Only the major criteria are necessary for the diagnosis of HRS, while the minor criteria are supportive. The diagnosis of HRS is one of exclusion and depends mainly on the level of serum creatinine, despite the fact it does not provide an accurate reflection of GFR in patients with cirrhosis[8]. Patients with cirrhosis with serum creatinine > 1.5 mg/dL have a GFR (estimated by inulin clearance) of < 30 mL/min, which represents one quarter of the normal GFR for healthy subjects of the same age[45]. HRS is a form of functional renal failure, therefore, the

Table 2 Diagnostic criteria of HRS

Major criteria
1 Chronic or acute liver disease with advanced liver failure and portal hypertension
2 Low GFR, as indicated by a serum creatinine of > 1.5 mg/dL or a 24-h creatinine clearance < 40 mL/min
3 Exclusion of shock, ongoing bacterial infection, volume depletion, and the use of nephrotoxic drugs
4 No improvement in renal function despite stopping diuretics and volume repletion with 1.5 L of saline
5 No proteinuria or ultrasonographic evidence of obstructive uropathy or parenchymal renal disease

Minor criteria[1]
1 Urine volume lower than 500 mg/day
2. Urine sodium lower than 10 mEq/L
3 Urine osmolality > plasma osmolality
4 Urine blood cells < 50 per high-power field
5 Serum sodium concentration lower than 130 mEq/L

[1]Only major criteria are necessary for the diagnosis of hepatorenal syndrome.

characteristics of urine are those of pre-renal azotemia with oliguria, low sodium concentration, and increased osmolality and urine to plasma ratio. These parameters are not considered essential for the diagnosis of HRS because they may overlap with different types of renal failure[44,45].

DIFFERENTIAL DIAGNOSIS OF ARF IN LIVER DISEASE

The differential diagnosis of ARF in advanced liver disease includes pre-renal failure, intrinsic renal failure and HRS (Table 3). The diagnostic evaluation relies upon clinical and laboratory data, including examination of urinary sediment and urinary chemistry, as well as appropriate ultrasonographic and radiological investigations[3,14,20]. Renal biopsy generally is not necessary for the diagnosis of ARF in liver disease, but is useful in excluding an intrinsic renal disorder[3]. A history of gastrointestinal hemorrhage, vomiting or diarrhea, exposure to nephrotoxic medication, or features suggestive of sepsis may provide important diagnostic information. Arterial hypertension, which is an unexpected finding in patients with cirrhosis, suggests glomerulonephritis[46]. The course of renal response to fluid challenge or vasoconstrictor therapy can also help differentiate causes of acute azotemia in liver disease. Rapid improvement in renal function denotes pre-renal failure, whereas mild or no improvement represents ATN or HRS[39,44]. Vasoconstrictor agents such as terlipressin or noradrenalin can sometimes be used to differentiate HRS and ATN, with improvement of GFR in favor of HRS[47,48]. Urine indices such as osmolality, sodium concentration, urine:plasma osmolality ratio (U/Posm) and urine:plasma creatinine ratio (U/Pcreat), are useful theoretical tools for differential diagnosis of the three principal causes of ARF in liver disease. However, in reality apart from urinary sediments these are often not clear cut.

Duplex Doppler ultrasonography, is a sensitive method of assessing intrarenal hemodynamics in patients with stable cirrhosis and ascites, in whom the renal artery resistive index is significantly increased and correlated with GFR and plasma renin activity[49]. However, this method is only useful in stable cirrhosis and may not be applied to acute situations.

Table 3 Differential diagnosis of ARF in advanced liver disease

	Prerenal failure	Intrinsic renal failure	HRS
Urine sodium	< 10	> 30	< 10
U/Pcreat	> 30:1	< 20:1	> 30:1
U/Posm	UO > PO	UO = PO	UO > PO
Urine sediment	Normal	Casts, cellular debris	Unremarkable
History disease	Profound volume	Volume contraction	Advanced liver disease
Clinical course (renal response)	Contraction	Nephrotoxic agent sepsis	Tence ascites
Fluid challenge	+	-	-
Vasoconstriction	±	-	+
Ultrasound	Elevated resitive index	Elevated resitive index	Elevated resitive index

MANAGEMENT OF ARF IN LIVER DISEASE

Management of ARF in liver disease should follow the same general principles as for the management of renal failure of any etiology, as well as specific measures for the liver disease. Combined ARF and ALF should ideally be managed in an intensive care or high-dependency setting. Initial management comprises correction of life-threatening abnormalities such as hyperkalemia, hypoglycemia, severe blood gas abnormalities, gross fluid overload and coagulation disorders, which may lead to bleeding and worsening renal function[50]. Although bleeding problems in patients with chronic liver failure are much less than previously thought, as a result of normal thrombin generation, renal failure superimposed on liver failure may have a negative impact on bleeding diathesis and worsening of hemorrhage[51].

Potential nephrotoxic drugs should be discontinued if possible, diuretic therapy interrupted, and infusion of crystalloid or colloid solutions commenced, based on clinical assessment and hemodynamic monitoring. In ALF complicated by intracranial hypertension, in addition to general measures to treat cerebral edema, early continuous renal replacement therapy (CRRT) may be considered, especially when patients are oligo-anuric and are taking mannitol[5,6].

In patients with chronic liver disease, management of ARF must focus on pre-renal failure, HRS and ATN. Upper gastrointestinal hemorrhage needs transfusion of plasma expanders and packed red blood cells, while measures are being taken to identify and treat the bleeding focus[12]. Intestinal and renal fluid losses should be replaced with appropriate fluid. Evidence of sepsis should be meticulously sought and a non-nephrotoxic broad-spectrum antibiotic regimen commenced, regardless of the etiology of sepsis. Current literature does not support the role of low-dose dopamine in the prevention and treatment of sepsis-induced renal vasoconstriction and

failure[52]. Vasopressin and terlipressin provide adequate splanchnic vasoconstriction and have been used not only in patients with cirrhosis and HRS, but also in sepsis in resistant cases[53].

Many different therapeutic approaches have been proposed for the management of HRS[2,13,27,38]. Unfortunately, most treatment measures result in only transient beneficial effects on renal function, and are not consistently associated with improvement in patient survival. Liver transplantation remains the definitive treatment for HRS, but is associated with higher hospital mortality compared to those without HRS who are treated with transplantation[54]. Thus, every attempt should be made to prevent this severe complication or reverse it when managing patients with cirrhosis and ascites. In recent years, new treatment strategies such as the use of vasoconstrictor drugs, along with plasma volume expansion, or insertion of TIPS, have shown some promise[20]. These treatments may prolong survival time and, therefore, act as a bridge to liver transplantation in these patients. Vasoconstrictors used for HRS include vasopressin analogues (ornipressin and terlipressin), somatostatin analogues (octreotide), and alpha-adrenergic agonists (midodrine and noradrenalin)[20]. In type 1 HRS terlipressin in combination with albumin has shown to result in greater improvement in renal function compared to terlipressin alone[10,53]. Pharmacological treatment, when combined with interventional techniques, such as transjugular intrahepatic portosystemic shunt (TIPS), may further improve renal function in HRS[27,55]. However, TIPS is frequently associated with significant side effects, particularly hepatic encephalopathy and impairment of liver function, and its role in the management of HRS needs to be established by prospective, controlled investigations[56].

The molecular adsorbent recirculating system (MARS) has been used in the treatment of acute decompensation of chronic liver disease (ADCLF), ALF and HRS[57]. This liver support system utilizes either intermittent (6-8 h daily) or continuous hemodialysis with dialysate enriched with 20% human serum albumin as a means to remove albumin-bound toxins (bilirubin, bile acids, fatty acids, tryptophan, aromatic amino acids, and copper). The first randomized trial of MARS evaluated 13 ADCLF patients with type 1 HRS. Five patients treated with hemodiafiltration alone died within 7 d, whereas three of eight patients treated with MARS were alive at 7 d, and two of eight were alive at 30 d[58]. Another recent randomized control trial, which included 24 patients with ADCLF, showed improvement in hyperbilirubinemia and hepatic encephalopathy and 30-d survival in patients treated with MARS. There was also improvement in renal function in the MARS group[59]. Until now, MARS has shown no benefit in improving survival in acute exacerbations of chronic liver failure. Likewise, MARS in the context of ALF has not been studied in control trials.

CRRT

Theoretically, the indications for CRRT in advanced liver disease and renal failure should be similar to those for general population. However, in view of the underlying disease, renal support should only be provided to those with a clear goal of hepatic management and a potential positive outcome i.e., the possibility of hepatic recovery or liver transplantation[60,61]. Measures such as volume expansion with albumin and the use of terlipressin should be tried before considering RRT. In fulminant hepatic failure (FHF), CRRT has become a major part of the routine management. Although there are no randomized trials to prove its efficacy, it can be assumed that it has contributed in part to the improvement in mortality. The continuous form provides greater hemodynamic, and more importantly, greater intracranial pressure ICP stability than the intermittent forms[62]. Continuous techniques (hemofiltration/hemodiafiltration) are preferred since they are associated with greater cardiovascular stability and allow gradual fluid removal, which can be adapted to actual needs and the infusion volume required for drug therapy and nutritional support[61].

PREVENTION OF RENAL FAILURE IN ADVANCED LIVER DISEASE

Two different strategies can be used to prevent HRS. The first is to perform liver transplantation in patients with cirrhosis and ascites before HRS develops. The identification of factors associated with a high risk of developing HRS and the use of duplex Doppler ultrasonography to assess the renal artery resistive index in the follow-up of these patients may be useful for this purpose[9,24,49]. The second strategy is to prevent the development of renal impairment in patients by avoiding the precipitating factors i.e., prompt management of bleeding and infection. A recent study has indicated that the development of HRS in patients with SBP can be effectively prevented by the addition of albumin to antibiotic (cefotaxime) therapy (1.5 g/kg human albumin intravenously at the time of diagnosis of the infection and 1 g/kg intravenously 48 h later). The proportion of patients who developed HRS and the in-hospital mortality was significantly lower in the cefotaxime-plus-albumin group that in the cefotaxime alone[63]. The beneficial effect of albumin is probably related to its ability to prevent circulatory dysfunction and subsequent activation of vasoconstrictor systems that occur during infection[63].

ARF POST LIVER RESECTION AND LIVER TRANSPLANTATION

Liver transplantation is the optimal treatment for patients with end-stage liver disease and ALF. The immediate outcome of orthotopic liver transplantation (OLT) is dependent on several factors, including pretransplant renal function and hemodynamic conditions in the operative and postoperative periods[63]. The prevalence of renal insufficiency in patients before transplantation varies from 10% to 20%, although many of these patients may have HRS, which is potentially a reversible condition.

Pretransplant renal dysfunction is a poor prognostic marker[64]. The reason for the poorer prognosis of these patients may be related to the persistence of the hyperdynamic circulation after liver transplantation, and to the fact that these patients seem to be more susceptible to damage from immunotherapy with cyclosporine or tacrolimus[65]. Additionally, pretransplant renal failure increases the incidence of postoperative sepsis, the need for pre- and postoperative dialysis, the number of days spend in the intensive care unit, and short-term graft and patient survival rates[64,66]. Renal failure is a frequent complication after OLT. It is usually acute, appears early after transplantation, and has an unfavorable effect on prognosis of liver transplant patients. The reported incidence ranges from 12% to 61%, according to the criteria used for defining ARF (serum creatinine ranging from 1.5 to 3 mg/dL or higher)[66,67]. Post-transplant ARF is usually caused by ATN due to perioperative complications (circulatory instability, duration), sepsis, repeated rejection, calcineurin-mediated renal vasoconstriction or nephrotoxic drugs (e.g., aminoglycosides and amphotericin B).

Prevention of renal failure after liver transplantation is not easy. The benefit of RRT with continuous venovenous hemodialysis before and after liver transplantation has been established[68]. Administration of aprotinin, an antifibrinolytic agent may be of benefit in the prevention of renal failure in adult patients undergoing OLT, by reducing intraoperative blood loss. Despite its potential nephrotoxic side effects, the administration of regular doses of aprotinin does not lead to a higher incidence of renal failure in these patients[69].

Treatment of HRS prior to OLT could also be beneficial for the prevention of ARF[63,66]. Finally, a delay in the introduction of nephrotoxic immunosuppresive drugs could be helpful in the prevention of post-transplant ARF, especially in high-risk patients.

CONCLUSION

Acute renal dysfunction is common in patients with acute and chronic liver disease. The presence of renal failure in this group of patients significantly affects mortality. Several advances have occurred in the management of this complication in the last decade. Improvement in the management of ARF in ALF is reflected in our better understanding of the disease process and better hemodynamic and renal replacement support. In advanced liver disease complicated by liver failure, terlipressin and early formation of TIPS have shown some promise. The role of MARS, bioartificial liver support, high-volume hemofiltration, and therapeutic plasma exchange, are still unclear and need further evaluation. Finally, as liver transplant is the definitive treatment, further education to increase the organ donation pool may be the best way forward.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Novel genetic markers in inflammatory bowel disease

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Abstract

Genetic factors play a significant role in determining inflammatory bowel disease (IBD) susceptibility. Epidemiologic data support genetic contribution to the pathogenesis of IBD, which include familial aggregation, twin studies, racial and ethnic differences in disease prevalence. Linkage studies have identified several susceptibility genes contained in different genomic regions named IBD1 to IBD9. Nucleotide oligomerization domain (NOD2) and human leukocyte antigen (HLA) genes are the most extensively studied genetic regions (IBD1 and IBD3 respectively) in IBD. Mutations of the NOD2 gene are associated with Crohn's disease (CD) and several HLA genes are associated with ulcerative colitis (UC) and CD. Toll like receptors (TLRs) have an important role in the innate immune response against infections by mediating recognition of pathogen-associated microbial patterns. Studying single-nucleotide polymorphisms (SNPs) in molecules involved in bacterial recognition seems to be essential to define genetic backgrounds at risk of IBD. Recently, numerous new genes have been identified to be involved in the genetic susceptibility to IBD: NOD1/Caspase-activation recruitment domains 4 (CARD4), Chemokine ligand 20 (CCL20), IL-11, and IL-18 among others. The characterization of these novel genes potentially will lead to the identification of therapeutic agents and clinical assessment of phenotype and prognosis in patients with IBD.

Key words: Genetic; Inflammatory bowel disease; Human leukocyte antigen; Nucleotide oligomerization domain; Toll like receptors; Susceptibility

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INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD) which are characterized by chronic illness of unknown etiology; however, its development is influenced by genetic, environmental and immunological factors[1].

Epidemiological studies suggest that genetic susceptibility is a major contributing factor to IBD. Molecular data from total genome scans and from candidate gene studies have led to the identification of genetic determinants of susceptibility and disease phenotype of UC and CD. The primary goal of genetic research is to identify genetic variants within specific genes which could modify homeostasis and increase disease susceptibility. There is growing attention to the innate immune response and the interaction between genetic factors and bacterial flora, or pathogen-associated molecular patterns in order to understand the contribution of environmental factors to disease susceptibility, as well as the phenotype based on a more precise molecular basis of disease pathogenesis. Clinical impact of the genetic findings has helped in understanding the heterogeneity of IBD in location, age at onset, clinical course and predicting response to conventional treatment.

GENETIC MODEL

Clinical and epidemiological data do not support a simple Mendelian model of inheritance for IBD. In its place CD and UC are considered to be complex polygenic diseases. Two major methods for identification of genes in complex multifactorial diseases are used, the positional cloning method based on association studies and the candidate gene analysis. Linkage analysis allows scanning of the whole genome studying the co-segregation of the disease with a marker within families, constituting an important method where allele sharing between affected sibling pairs is used. An alteration of the observed ratio of sharing contrasting the expected is interpreted as evidence for linkage with a particular marker. On the other hand, the candidate gene analysis attempts to determine the

Table 1 Major regions and genes in IBD

Region	Localization	Involved genes
IBD1	Chromosome 16	NOD2/CARD15, IL-4R, CD11B
IBD2	Chromosome 12	Vitamin D receptor (VDR), STAT6, Interferon γ, β7 integrine.
IBD3	Chromosome 6	Major histocompatibility complex (MHC): Class Ⅰ, Ⅱ, Ⅲ.
IBD4	Chromosome 14	T- Lymphocyte receptor (TCR) and Leukotriene B4
IBD5	Chromosome 5	Organic cations transporter (OCTN), *Drosophila long disc homologue gene 5* (DLG5), *Multidrug resistant gene* (MDR1), IL-6, CD14
IBD6	Chromosome 19	Thromboxane A2, Leukotriene B4, ICAM-1
IBD7	Chromosome 1	Transforming growth factor Beta (TGFβ), TNFα receptors.
IBD8	Chromosome 16	Under research
IBD9	Chromosome 9	CCR-5, CCR9, IL-12

NOD2/CARD15: Nucleotide oligomerization domain 2/Caspase-activation recruitment domains 15; STAT6: signal transducer and activator of transcription-6; ICAM-1: intracellular adhesion molecule 1; CCR5: CC-chemokine receptor5.

importance of specific genes in disease pathogenesis, using case-control cohorts or trios of affected progeny with both parents. The allelic frequencies or the transmission of a single-nucleotide polymorphism (SNP) towards affected progeny is studied and the differences between patients and controls might point towards the implication of a particular gene in the pathogenesis of the disease under investigation. It also includes positional candidate genes which are found in areas of linkage defined by genome screening. As CD and UC are likely to share some susceptibility genes, it has been proposed a genetic model of UC and CD where the two are polygenic disorders, sharing some susceptibility *loci*, but differing in others[2]. Linkage studies support this concept because some *loci* appear to interfere with susceptibility to IBD, which have also been implicated in the abnormal immune response and those susceptibility genes could interfere with the disease phenotype such as extension, need for procto-colectomy, extraintestinal manifestations, as well as the response to different treatments. From most of the genome-wide scans performed in IBD, a number of susceptibility regions on several chromosomes have been found[3-6] and according to their initial date of reporting and independent confirmations, the regions on such chromosomes have been renamed as IBD1-9 (Table 1).

IBD1

Accurate mapping of the IBD1 locus has led to identification of the underlying gene called the NOD2/Caspase-activation recruitment domains 15 (CARD15) gene located on the pericentromeric region on the long arm of chromosome 16 (16p12.3) extending to 16q13[7-10]. Several studies have demonstrated the identification of NOD2/CARD15 gene within the IBD1 locus as a susceptibility gene in CD, suggesting that approximately 25%-30% of the genetic susceptibility in CD can be explained by mutations in NOD2/CARD5 though most of these studies have shown no association between NOD2 mutations and susceptibility to UC[11,12]. Thirty non-conservative polymorphisms have been identified within the gene that are associated with CD and only three are common (Arg702Trp, Gly908Arg and Leu1007insC). The three common variants, however, account for approximately 82% of the mutated alleles[13]. Nevertheless, these mutations seem to have different effects on the risk of developing CD: Arg702Trp, Gly908Arg and a deletion in the last 33 aminoacids Leu1007finsC, which are present in 43% of the patients with CD (10%-30% is heterozygous and 2%-15% is homozygous for these mutations)[8-10]. These variants in NOD2 have been associated with certain clinical features of CD[14]. The NOD2 contribution seems to be stronger in Ashkenazi Jewish population who has a higher frequency of the Gly908Arg NOD2 variant. In relation to phenotypic expression and translation into the clinic, some associations between NOD2 mutations and earlier-onset disease in adult populations, fistulizing disease, fibrostenosing disease behavior and increased risk of need for surgery in children have been described[15-18].

CARD gene codes for a protein expressed in several cells of innate immunity, epithelial cells and Paneth cells[11,12]. This protein consists of two N-terminal CARD, a central nucleotide-binding domain and a C-terminal leucin-rich-repeat region (LRR). It has been reported that CARD15 is implicated in the recognition of a bacterial product peptidoglycan-derived muramyl dipeptide (MDP) that enters into the cytosol via a transporter protein hPepT1 and interacts with the LRR of NOD2. Mutations within the leucine-rich region are associated with CD, as mutations within the nucleotide-binding domain are associated with granulomatous diseases[19]. Through the recognition of MDP, secretion of alpha-defensins is stimulated for protection against microbial invasion. In CD patients, a diminished expression of alpha-defensins has been found in those carriers with NOD2 mutation[20,21]. It is known that through this recognition of bacterial products the nuclear factor kappa B (NFκB) activation is regulated[10,22].

Evidence show that NOD2 protein acts as an important regulator of NFκB activation in response to the Toll-like receptor (TLR) 2 activation system leading to its down regulation[23,24]. However in carriers of mutant protein, this process does not occur and proinflammatory cytokines are produced with a Th1 profile[25,26].

IBD2

This region is located on chromosome 12 showing greater linkage evidence in UC compared with CD. A number of possible candidate genes have been investigated including signal transducer and activator of transcription-6 (STAT6), INFγ, metalloproteinase (MMP18), Vitamin D receptor (VDR) and β7 integrin family that could be associated with the susceptibility to IBD. Parkes *et al*[27] found that IBD2 appears to make a major contribution to UC susceptibility but has only a relatively minor effect on CD.

Vitamin D receptor

The Vitamin D receptor (VDR) is a member of a steroid receptor family and mediates the effects of the active metabolite 1.25 $(OH)_2$ vitamin D_3 by regulating

transcription of a number of different genes. It is synthesized by activated macrophages. It is expressed by monocytes and activated B and T lymphocytes. It activates monocytes and macrophages but suppresses lymphocyte proliferation and immunoglobulin production, and also inhibits transcription factor NFκB, and the production of IL-2, IL-12 and interferon γ. 1.25 $(OH)_2D_3$ is the form of vitamin D that binds to the VDR and inhibits experimental autoimmunity[28,29]. Vitamin D deficiency and VDR deficiency have been shown to exacerbate chronic IBD in IL-10 knock out mice[29,30]. Absence of the VDR results in mice that are extremely susceptible to chemical injury in the gut[30]. The linked SNPs found at the 3' end of VDR are: *Bsm*I, *Apa*I, *Taq*I, and the exon 2 splice site *Fok*I polymorphism[31,32]. *Fok*I polymorphism has been associated with osteoporosis, *Taq*I polymorphism with the risk of prostate cancer[33] and recently homozygotes for the *Taq*I allele have been shown to have altered susceptibility to a variety of infectious diseases[34]. Simmons *et al*[35] studied 403 European Caucasian patients with IBD, and found significantly more homozygotes for the *Taq*I polymorphism among patients with CD than in patients with UC or controls, providing evidence for a genetic association between CD susceptibility and a gene that lies within one of the candidate regions determined by linkage analysis. Dressner-Pollak *et al*[36] found that *Bsm*I VDR gene polymorphism is associated with increased susceptibility to UC in Israeli Ashkenazi patients with UC contrasting with *Taq*I polymorphisms that favor susceptibility to CD. It seems that in the absence of the VDR, inflammation in the gut is increased, colonic epithelial cell proliferation is dysregulated, and the host tissue fails to satisfactorily maintain gastrointestinal integrity following chemical insult. These data identify vitamin D as a key regulator of gastrointestinal homeostasis and an important player in regulation of the innate immune response.

IFN-γ

IFN-γ seems to be specifically important in CD pathogenesis as suggested by case control studies that showed elevated levels of IFN-γ production in the mucosa in patients with CD, but not in UC[37]. Data indicate that patients with relapsing perianal fistulizing disease have an increased production of IFN-γ measured by *in vitro* cell cultures[38]. Cytokine genotyping study showed that IFN-γ (+874T/A) polymorphism is found in an increased proportion of patients with fistulizing CD[39] probably related to the reduction of tissue repair and migratory potential in fibroblasts apparently influenced by IFN-γ in CD patients[40].

IBD3

Major histocompatibility complex (MHC) genes are located in this region, specifically on the short arm of the human chromosome 6.

With a candidate gene approach the MHC is the most extensively studied region. Two meta-analyses have been carried out to scan for IBD regions that are common for all populations[5,41] in which the highest evidence for linkage to IBD was achieved at the IBD3 locus. There are 3 classes of MHC genes: I, II and III. The antigenic recognizing process in T-lymphocytes from the antigen presenting cells is achieved through the antigenic recognition associated with the MHC gene product[42].

HLA class I

Some not classical genes related to the class I genes such as MHC class I chain-related gene A (MICA) and MHC class I-related chain B (MICB), are expressed in the basolateral cells in the gastric epithelium, fibroblasts, endothelial and dendritic cells. It is known that its expression rises during viral and bacterial infections[43]. Some genetic studies in patients with IBD have found associations with MICA-A6 and HLA-B52 in Japanese patients with UC[44], MICA*010 and HLA-B*1501 in English patients with fistulous CD[23]. MICA and MICB bind to an activating receptor natural killer group 2D (NKG2D) which is expressed on NK cells, T cells and macrophages and the interactions between these receptors may directly stimulate cell cytotoxicity as well as providing costimulation for NK and T cell activation. Several MICA alleles have been shown to alter the binding affinity with NKG2D suggesting they may exert a functional effect on immune activation. In contrast to HLA class II, HLA class I genes show a weak and inconsistent role in IBD. The functional significance of these polymorphisms and the nature of selective forces maintaining them are still being elucidated.

HLA class II

Class II genes are located on the centromeric pole of the short arm of the human chromosome 6 and include HLA-DR, DP and DQ *loci* expressed in a dimeric glycoprotein only in macrophages, activated T-lymphocytes, B-lymphocytes, dendritic, epithelial and endothelial cells, playing a central role in the immune response. Polymorphisms in these molecules are concentrated around specific pockets of the binding groove that interact with critical side-chains or anchor residues of peptides. The different HLA molecules may bind preferentially to different peptides, or bind the same peptide with varying affinities. In IBD the molecular mimicry may exist between the peptides derived from bacterial luminal flora and from self antigens present in the gut, leading to the generation of auto reactive T cells and contributing to disease pathogenesis. The mechanism of cross reactivity is supported by the identification of murine MHC-restricted CD4+ T cells reactive to enteric bacterial antigens that are able to induce colitis by adoptive transfer[45].

In a meta-analysis made by Stokkers *et al*[46], positive associations between UC and HLA-DR2, HLA-DRB1*1502, HLA-DR9 and HLA-DRB1*0103 were found. A study from Mexican population found that HLA-DRB1*0103 allele was associated with UC and its severe manifestations such as colectomy and pancolitis, while HLA-DRB1*15 allele was only associated with pancolitis in Mexican patients with UC.

HLA class III

These genes are located on the 1100 kb section between class I and II genes inside the MHC, and contain about 70

genes. The complement gene block is inherited as a genetic unit known as complotype. Each complotype codifies for the synthesis of complement classic pathway C2, C4A, C4b factors, and alternative pathway B factor, which may suggest that alterations within the region might affect the host's defense system and introduce a complement deficiency. This raises attention when TNFα is thought to play an important role in the pathogenesis of IBD, acting as a potent proinflammatory cytokine with elevated serum and tissue levels in patients with IBD[47-49], and evidence show that there are specific genetic polymorphisms involving TNFα that influence the amount of cytokine produced. Bouma *et al*[50] and Louis *et al*[51] studied the allelic frequency of TNFα gene polymorphisms at -308 position finding that polymorphism in allele 2 was decreased in UC patients as compared to normal controls. It was also found that patients with pancolitis had increased frequency in the TNF-C haplotype[52]. In a Mexican population with UC, the presence of TNF*2 allele was associated with the presence of this disease as compared with healthy subjects (23.7% *vs* 3%, $P = 0.00002$; OR = 10.1; 95% CI = 2.69-26.8)[53]. In Mexican patients with UC, an association was found between complotype SC30 (Bf*S-C2*C-C4A*3-C4B*0) and UC[54], which might suggest that activation of complement system could interfere with the disease pathogenesis.

IBD4

This locus is located on chromosome 14. Evidence for linkage to the adjacent D14S261 and D14S283 *loci* on chromosome 14q11-12 satisfied criteria for confirmed linkage and this region was designated IBD4 locus[55]. Vermeire *et al*[56] in a genome wide scan in a 149 Belgian IBD affected families cohort, demonstrated the existence of IBD4 on 14q11 as a susceptibility *loci*. The IL-25 gene is located within this susceptibility region at 14q11.2. The IL-25 gene is located within this susceptibility region at 14q11.2.

IL-25

Interleukin-25 (IL-25) is a newly identified proinflammatory cytokine that has been shown to promote Th2 responses by inducing cytokines such as IL-4, IL-5 and IL-13, implicated in the initiation of type 2 cytokine-dependent immunity to gastrointestinal infection and limiting proinflammatory cytokine production and chronic intestinal inflammation. IL-25-deficient knockout mice failed to develop a type 2 immune response or eradicate infection. Moreover, chronically infected IL-25 (-/-) mice developed severe infection-induced intestinal inflammation identifying a role for IL-25 in limiting pathologic inflammation at mucosal sites in the gastrointestinal tract[57]. Still more evidence is needed to conclude a precise role of this gene in the IBD susceptibility.

IBD5

The IBD5 locus on chromosome 5q31-33[58] contains the cytokine cluster and is a candidate region for IBD. The IBD5 risk haplotype has been associated with CD, although there have been some suggestions of a weak association with UC as well. Phenotypically this locus has been associated with earlier onset of disease as well as perianal disease[59-62].

Carnitine/Organic cation transporter genes

The organic cation transporter genes OCTN1 and OCTN2 are within a single haplotype block (block 7) of the IBD5 locus and some mutations have been reported within these: L503F (rs1050152) and G-207C (rs2631367) in the *SLC22A4 (OCTN1)* and *SLC22A5 (OCTN2)* genes, respectively, which are associated with the development of CD and also an association with susceptibility to UC has been reported[63]. The presence or combination of these mutations constitutes the TC haplotype, which is associated with ileal, colonic and perianal disease[64,65]. Associations between TC haplotype variants and CD affected sites have been shown in genotype-phenotype studies[66,67]. According to some studies, 1672C→T missense substitution in SLC22A4 and the -207G→C transversion in the SLC22A5 promoter contribute to disease susceptibility by impairing OCTN activity or expression respectively[68]. The risk associated with the OCTN-TC haplotype seems to be only observed in homozygotes and not in heterozygotes[60,63,69], so the carriage of the homozygous OCTN-TC haplotype is likely to be associated with a higher relative risk for colonic disease. The association of the OCTN polymorphisms with CD phenotypes shows a higher frequency of the OCTN-TC haplotype in patients with colonic involvement compared with exclusive ileal disease[60]. It has been reported a moderate increase in the frequency of the TC haplotype among patients without fistulas or stenosis and this is compatible with the negative association with ileal involvement, showing a tendency towards a lower-frequency of ileocecal resection in the presence of at least one OCTN-TC haplotype and might explain the absence of colonic involvement[64]. The impaired eradication of luminal pathogens results in a persistent infection which may constitute a possible mechanism causing IBD.

Drosophila long disc homologue 5 gene

Drosophila long disc homologue 5 gene (DLG5) on chromosome 10q22-23 is a member of the membrane associated guanylate kinase gene family which encodes cell scaffolding proteins and seems to play a role in the maintenance of intestinal epithelial cells, and its mutations have been involved in a rise in intestinal permeability[70]. DLG5 is a widely expressed protein found in many tissues such as the placenta, small bowel, colon, heart, skeletal muscle, liver and pancreas and it is important in signal transduction and epithelial cell integrity. Four haplotypes have been identified associated with IBD in a European cohort[71]. Haplotype A is characterized by the presence of an insertion of thirteen pairs in exon 26. It has been shown to be protective in some case control studies[72], however it is substantially undertransmitted in people with IBD[62]. The haplotype characterized by the haplotype-tagging SNP G113A called Haplotype D, was found substantially overtransmitted in patients with IBD controversially contradictory with another[73] Belgian study where the D haplotype involving the 113A variant was shown to be undertransmitted in flamish patients with

IBD. These apparently contradictory results might yet be compatible with the possibility that DLG5 has a small effect in IBD with heterogeneity in its effect.

ATP-binding cassette or multidrug resistant gene

The multidrug-resistance (MDR1) gene is located on the long arm of chromosome 7 and consists of 29 exons. The total length is 209 kb and 6326 bp. Its product, the P-glycoprotein (Pgp), a member of the ATP binding cassette family, is an integral membrane protein which functions as an energy-dependent efflux pump and reduces the intracellular concentrations of toxins and xenobiotics[74]. Studies show evidence for natural single nucleotide polymorphisms (SNPs) of MDR1 gene and their effects on drug efficiency, toxicity, distribution, absorption and elimination. Two main polymorphisms or variants of this gene have been described, C3435T and G2677T which are associated with IBD in some populations[75,76] and have also been related with the expression of glycoprotein P-170. Variant C3435T was related with the presence of pancolitis in patients with UC in Scotland[77]. However, the frequency of SNPs is low and is different among populations, with the exception of three SNPs in exon 12 (C1236T), exon 20 (G2677T/A) and exon 26 (C3435T), and some of them are correlated with different diseases and clinical characteristics[78]. Glucocorticoid is a potent inhibitor of the T cell activation and a highly effective treatment for IBD[79]. Over-expression produces three molecular mechanisms of glucocorticoid resistance: increase of P-gp and decrease of cytoplasmatic glucocorticoid, dysfunction at the level of glucocorticoid receptor and activation of NFκB, resulting in inhibition of glucocorticoid receptor transcriptional activity. Cucchiara *et al*[80] investigated the predisposition and response to medical therapy of TNFα and MDR1 genes in 200 pediatric patients with CD and 186 UC patients and 347 adults as a control. The 308A allele of the TNF-α gene was increased in both patients with CD and UC, strongly suggesting this polymorphism carries a significant reduction in response to steroid therapy.

IL-6 (-174G/C) polymorphism

IL-6 is a well-studied IBD candidate gene and its polymorphism has been associated with the site of disease in CD. IL-6 levels are higher in patients with active CD as compared to patients with active UC and normal controls[81,82]. A study from Cantor *et al*[39] demonstrated a relationship between IL-6 genotype and the site of CD, showing that patients with the high producer of IL-6 genotype were more likely to have colonic CD. In CD patients IL-6 concentrations also correlate with the disease activity, response to treatment and rate of relapse.

IBD6

In a Canadian linkage scan, a linkage peak of genome-wide scan on chromosome 19p was identified and appeared to confer susceptibility to both CD and UC[61]. Two independent genome-wide linkage studies also determined evidence for linkage to this region and two other meta-analyses of all published genome-wide scans[5,41,83] identified evidence that supports the existence of a locus conferring susceptibility to IBD in chromosomal region 19p, currently known as the IBD6 locus. In order to identify IBD susceptibility alleles in the 19p region two candidate genes DDXL and intracellular adhesion molecule 1 (ICAM-1) were examined in a case-control study with CD and UC patients but no association with either UC or CD was found in 3 single nucleotide polymorphisms in DDXL gene, however a significant association was found between ICAM-1 K469 homozygosity and CD as well as E469 and fistulating disease[84].

IBD7

Located on the short arm of chromosome 1, IBD7 is thought susceptibility genes are residing in this locus. One of these codifies for the transforming growth factor beta 2 (TGF-beta 2) which is a cytokine present in human and bovine milk and plays a critical role in the development of tolerance, prevention of autoimmunity, and in anti-inflammatory responses and is also a potent inhibitor of intestinal epithelial cell (IEC) growth and stimulates IEC differentiation[85-87]. McKaig and colleagues[88] studied the expression of TGF-beta isoforms in isolated and cultured primary human intestinal myofibroblasts from normal controls as well as from UC and CD patients, and determined the responsiveness of these cells to TGF-beta isoforms. Proliferation of myofibroblasts in CD patients was significantly greater than that of myofibroblasts derived from normal and ulcerative colitis tissue, suggesting that it may be related to the development of intestinal strictures, seen frequently as a major feature in CD. The anti-inflammatory attributes of TGF-β3 may be evidenced in a study of children with active intestinal Crohn's disease, who were treated with an oral polymeric diet rich in TGF-β2 as the sole source of nutrition for eight weeks and it was associated with mucosal healing and a down-regulation of mucosal pro-inflammatory cytokines mRNA in both the terminal ileum and colon[89]. However, further investigation on this locus is needed to determine the level of significance related to the pathogenesis of IBD.

IBD8

This gene is located on the short arm of human chromosome 16. There has been evidence of a second chromosome 16 locus (IBD8) independent of NOD2 that overlaps IBD1 on the pericentromeric short arm[90], but yet no studies have been performed for the identification on this locus.

IBD9

The CC-chemokine receptor 5 *(CCR5)* gene located on chromosome 3p21 coincides with this IBD susceptibility locus identified by genome-wide scanning[91]. The CCR5 is the receptor for regulated and normal T-cell expressed and secreted (RANTES), a natural pro-inflammatory cytokine. A 32-bp deletion (A32) in the CCR5 gene results in a nonfunctional receptor found with a high

frequency in Caucasians. They found an association between CCR5delta32 homozygosity and the presence of anal lesions in CD patients with statistical significance[92]. Several genes located in these regions are still under research (Table 1).

GENES INVOLVED IN THE INNATE IMMUNE RESPONSE

Toll like receptors

Rising evidence suggests an essential role of the enteric bacterial flora in the pathogenesis of IBD. Rather than a passive barrier, the intestinal epithelium is an active participant in the mucosal immune response through its expression of proinflammatory genes, secretion of inflammatory cytokines, and recruitment of inflammatory cells in response to pathogenic bacteria and their products[93]. IBD has been increasingly thought to result from an aberrant interaction between the environment and the genetically susceptible host. Specifically, several lines of evidence point to a deregulation of the immune response to a commensal or uncharacterized pathogenic bacterium in the gut[94]. Animal models have demonstrated that genes involved in the regulation of the immune response are likely to play a crucial role in the genetic predisposition to IBD[95]. The family of Toll-like receptors *(TLR)* recognizes pathogen-associated molecular patterns and activates signal transduction pathways of the innate immune response genes including NFκB[95]. The SNPs involved in bacterial recognition are becoming essential in understanding individual responses to bacterial components and defining genetic backgrounds at risk of IBD.

Toll-like receptor 4: The toll-like receptor 4 (TLR4) gene is located on the long arm of human chromosome 9 and it identifies lipopolysaccharides (LPS) on gram-negative bacteria. It has been found strongly upregulated in IBD, and it binds to LPS together with CD14 and by internalization prevents inappropriate NFκB activation[96]. The TLR4 Asp299Gly polymorphism has been associated with CD and UC in a Belgian study[97]. On the other hand this SNP was exclusively related to CD in other series[97-102] and TLR4 polymorphism Thr399Ile was exclusively associated with UC in others[99]. A lipid A-mimetic CRX-526 with antagonistic activity for TLR4, is known to block the interaction of LPS with the immune system, therefore, CRX-526 can prevent the expression of proinflammatory genes stimulated by LPS *in vitro*. This disturbed activation of the innate immune system by bacterial antigens may be crucial in some patients with IBD.

TLR1, TLR2, and TLR6: Pierik *et al*[100] studied the nonsynonymous polymorphisms in other TLR genes in IBD. They found no SNP was involved in disease susceptibility, and a number of variants influenced the disease phenotype, however, they found a positive association between TLR1 R80T and TLR2 R753G and pancolitis in UC. TLR2 and its cofactors TLR1 and TLR6 are involved in the initial immune response to bacteria and recognition of peptidoglycan. This TLR2 is required for recognition of Gram-positive and mycobacterial pathogen-associated molecular patterns (PAMPs) including bacterial lipopeptide lipoteichoic acid (LTA), peptidoglycan (PGN) and the mutations associated are involved in severe mycobacterial infections[103-109]. Further studies have shown that combinations of TLR molecules are required for recognition of certain PAMPs and that specifically, combined expression of TLR2 and TLR6 is required for recognition of PGN[110-114].

It is suggested that TLR1 may be regulated diversely in inflammation to down-regulate or enhance the response to certain TLR2 ligands and that a relative absence of TLR2 protein expression may be important in preventing chronic proinflammatory cytokine secretion in response to commensal Gram-positive bacteria in the gut[95].

TLR5: *TLR5* gene is located on the short arm of human chromosome 1 and is responsible for recognizing a protein named flagellin which is found in intestinal bacteria[113]. Lodes *et al*[113] observed through serological studies a strong response to flagellin in multiple animal models of colitis and synergism has been identified between NOD2 and TLR5 signaling[114]. The dominant negative variant of TLR5 (TLR5-stop) seems to protect against the development of CD and results in significant reduction of IgA and IgE circulating antibodies against flagellin[115], suggesting that pharmacological blockade of TLR5 has potential in the treatment of CD.

NOD1/CARD4 gene

Located on chromosome 7p14, *NOD1/CARD4* gene is one of the three human NOD-LLR proteins that has similar structure to NOD2/CARD15, having only one CARD domain, a central NOD domain and a leucine rich repeat region (LLR). Its function is the recognition of gram negative bacterial products such as γ-glutamine diaminopimelic acid and plays a role in colonic epithelial defense against the intracellular pathogens *E.coli* and *Shigella flexeneri*. Its effector domain is associated with Ripk2 (a CARD-containing interleukin-1 beta converting enzyme-associated kinase) mediating NFκB activation. In a recent study of 556 patients with IBD (294 CD and 252 UC), an association between the variant rs695857 in nucleotides 30, 258 and 950 of NOD1 and the development of IBD was found. Another variant known as rs2907748 in nucleotides 30, 246 and 263 was also associated with the presence of UC and CD and even with the early onset of the disease (< 25 years)[116]. These genetic variants of NOD1 have shown to be associated with disease susceptibility supporting that impaired local immunity might influence bacterial proliferation and aberrant immune responses in the host.

CCL20

CC-chemokine ligand 20 (CCL20) gene is located on the short arm of human chromosome 2 and codifies for the CCL20 cytokine ligand, which is responsible for the chemoattraction of immature dendritic cells that express CCR6 receptor on the intestinal epithelium and on Peyer's plaques[117], and also attracts memory T lymphocytes.

Microarray analysis and PCR-RT quantification have shown a rise in the expression of mRNA from IBD biopsies with inflammation compared to normal biopsies[118]. A study made in Korean UC patients showed that the expression of CCL20 was significantly up-regulated in the peripheral blood mononuclear cells compared with those of normal healthy controls. Interestingly, untreated UC groups expressed higher levels of CCL20 mRNA than treated UC and normal control groups, therefore suggesting that CCL20 could be modulated by anti-inflammatory drugs[119].

Interleukin 11

IL-11 mediates anti-inflammatory effects and is able to downregulate LPS-induced NFκB activation. The IL-11 gene is therefore a good candidate involved in genetic predisposition to IBD. Klein *et al*[120] evaluated the role of IL-11 in IBD, finding decreased expression and a failure to downregulate NFκB expression that could play an important role in the pathogenesis of UC.

Interleukin 18

IL-18 is a pleiotropic cytokine that induces the production of IFNγ and also regulates Th2 cytokines. It seems to be an important cytokine involved in the pathogenesis of IBD, apparently because SNPs at the 5'-end of IL-18 gene might be closely related to the etiology of IBD. Takagawa *et al*[121] found that IL-18 gene promoter polymorphisms may be related to the extent of disease in UC patients.

Interleukin 23

IL-23 is a heterodimeric cytokine composed of a p19 subunit and the p40 subunit of IL-12. It is produced by macrophages and dendritic cells, and activates memory T cells. Interleukin-12 (IL-12) is composed of p35 and p40 subunits and acts as an important factor for the differentiation of naive T cells into T-helper type 1 CD4+ lymphocytes secreting interferon-gamma. Therefore it has been reported that IL-12 is crucial for T-cell-dependent immune and inflammatory responses through the use of IL-12 p40 gene-targeted mice and neutralizing antibodies against p40[122-127]. Apparently IL-12 is a key factor driving Th1 responses and IFN production in the initial phases of an immune response, but conversely IL-12 may play a subsequent immunoregulatory role in late-stage inflammation at a point when IL-23 strongly supports the inflammatory process. IL-23 induces the production of IL-17 by a unique subset of memory T cells. IL-17 is known to stimulate fibroblasts, endothelial cells, macrophages and epithelial cells to secrete multiple pro-inflammatory mediators[128] and the local production of IL-17 causes site-specific activation of inflammatory cells[129-132]. Dendritic cells found in the lamina propria of the small intestine were described as constitutively expressing IL-23[131], whereas IL-23 regulates a highly potent T cell-derived cytokine that has major actions on the immune system. IL-23 specifically stimulates memory CD4+T cells contrasting the IL-12 which is a stimulant for naive CD4+ T cells[129,130]. Studies with IL-23 deficient mice show that IL-23 is essential for the manifestation of intestinal inflammation and a dominant role for IL-23 over IL-12 in central nervous system and joint autoimmune inflammation has been described. These findings point to IL-23, but not IL-12, as the necessary mediator for organ specific autoimmune diseases development. Furthermore, the absence of IL-12 results in more severe disease, reflected in elevated and prolonged expression of proinflammatory cytokines. Yen and colleagues[132] reported that the activation of tissue-homing memory T cells by IL-23 is responsible for chronic inflammatory disease.

CONCLUSION

Genetic research in IBD has provided knowledge about the complexity and heterogeneity of the disease and started to correlate the interactions between genetic and environmental risk factors in IBD; however, the complex genetic background that allows the development of IBD is not fully understood.

Understanding the pathways in which genetic factors influence IBD will uncover pathogenesis of the disease, offer more accurate diagnosis and ultimately lead to the breakthrough of better new drugs and therapies. Most of the important advances toward understanding this process have been identification of specific genetic associations with IBD, which will shed new light on future research of IBD.

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TOPIC HIGHLIGHT

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Role of bacteria in the etiopathogenesis of inflammatory bowel disease

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Abstract

Increased numbers of mucosa-associated *Escherichia coli* are observed in both of the major inflammatory bowel diseases, Crohn's disease (CD) and ulcerative colitis (UC). A potential pathophysiological link between the presence of pathogenic invasive bacteria and genetic host susceptibility of patients with ileal CD is suspected. In CD patients, with increased ileal expression of the CEACAM6 molecule acting as a receptor recognized by type 1 pilus bacterial adhesin, and with the identification of mutations in the NOD2-encoding gene, the presence of pathogenic invasive bacteria could be the link between abnormal ileal bacterial colonization and innate immune responses to invasive bacteria. In a susceptible host, the sequential etiological steps of the disease induced by adherent-invasive *E. coli* (AIEC) are: (1) abnormal colonization *via* binding to the CEACAM6 receptor, which is overexpressed in the ileal mucosa of CD patients; (2) ability to adhere to and to invade intestinal epithelial cells, which allows bacteria to cross the mucosal barrier; (3) survival and replication within infected macrophages in the lamina propria; and (4) induction of tumor necrosis factor-α secretion and granuloma formation.

Key words: Adherent-invasive *Escherichia coli*; Crohn's disease; Inflammatory bowel disease; Ulcerative colitis

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INTRODUCTION

Idiopathic inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic disorders of the gastrointestinal tract that have a combined prevalence of approximate 150-200 cases per 100 000 population in Western countries[1]. Several lines of evidence suggest that bacteria play a role in the onset and perpetuation of IBD[2-6]. Intestinal bacteria are essential for the development of intestinal inflammation, and are required for the onset of inflammation in numerous knockout models of IBD[7-9]. The pathogenesis of CD is complex and consists of three interacting elements: genetic susceptibility factors such as NOD2/CARD15 and ileal CEACAM6 expression; priming by enteric microflora; and immune-mediated tissue injury[4,6,10-13]. The role of luminal bacteria in the pathogenesis of CD is strongly supported by observations showing that clinical symptoms of CD improve when luminal bacterial levels decrease following intestinal washes and antibacterial drug administration[14-16]. In addition, postoperative exposure of the terminal ileum to luminal contents is associated with increased inflammation in CD, and diversion of the fecal stream is associated with improvement[17].

Studies of luminal bacterial composition in patients with IBD, using culture and molecular biology techniques, have shown a decrease in the number of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* spp. and an increase in pathogenic bacteria such as Bacteroides and *Escherichia coli* (*E. coli*)[18-20]. Such dysbiosis induces a breakdown in the balance between putative species of protective *vs* harmful intestinal bacteria, and may promote inflammation[21,22]. Patients with IBD have higher numbers of mucosa-associated bacteria than control patients[18], and the generalized or localized dysbiosis observed is due to the presence of low numbers of normal bacteria, high numbers of unusual bacteria, and sometimes, a reduction in biodiversity. CD has features that might be the result of a microbial process in the gut. These include onset of infection in Peyer's patches and lymphoid aggregates, and the presence of ulceration, micro-abscesses, fissures, fistulas, granulomas and lymphangitis. Interestingly, the earliest lesions are aphthous ulcers in the intestine, which also occur in some viral and bacterial infections.

Although a number of organisms have been implicated in CD, only two agents, *Mycobacterium paratuberculosis* and *E. coli*, are presently being actively investigated. The theory that *M. paratuberculosis* has a role in CD has some attractive features[23]. Indeed, there are clinical similarities between Johne's disease, a spontaneous *M. paratuberculosis* infection in ruminants, and CD. *M. paratuberculosis* is detected at a greater frequency in CD than in control patients (UC patients and healthy subjects), by culture and polymerase chain reaction (PCR). This organism has been detected in blood and breast milk of patients with CD[24]. The high levels of *E. coli* colonizing the intestinal mucosa in CD patients strongly suggest that it plays a role in the etiopathogenesis of CD.

E. COLI ABNORMALLY COLONIZES ILEAL MUCOSA OF GENETICALLY PREDISPOSED IBD PATIENTS

Bacterial adhesion to intestinal epithelial cells is the first step in the pathogenicity of many organisms involved in infectious diseases of the gut. Adhesion enables the bacteria to colonize the mucosa and to resist mechanical removal from the intestine. Studies on the adherence properties of *E. coli* in CD have yielded the general conclusion that *E. coli* strains are able to adhere to various human cells or cell lines. Fifty-three to 62% of *E. coli* strains isolated from feces of CD patients were able to adhere to buccal cells, compared to only 5%-6% of those isolated from control subjects[25,26]. The comparison of the adhesive properties of *E. coli* strains isolated from the ileum of CD patients and controls has revealed that 80% of *E. coli* strains associated with the ileal mucosa of CD patients preferentially adhered to differentiated Caco-2 cells, which mimic a mature intestinal cell model[20]. This is consistent with the finding that in patients with CD, crypt epithelial cells, which correspond to immature cells, are rarely involved in early lesions[27]. In addition, a correlation between bacterial adhesion to intestinal cells and intestinal colonization has been observed[20]. The presence of high levels of bacteria creates a biofilm on the surface of the gut mucosa in patients with CD and UC[18]. When bacteriologic samples were taken during surgery for CD, *E. coli* was isolated more frequently from the intestinal serosa and mesenteric nodes of CD patients (27% and 33%, respectively) than from those of control subjects[28,29]. Increased numbers of mucosa-associated *E. coli* are observed in CD and UC[18-20,30-33]. Rectal mucosa-associated *E. coli* counts were also higher in active than in inactive UC and CD and controls, and clusters of *E. coli* were observed in the lamina propria in UC and CD specimens, but not in controls[34]. In a study to assess the predominance of *E. coli* strains associated with the ileal mucosa of CD patients, *E. coli* was recovered from 65% of chronic lesions (resected ileum) and from 100% of the biopsies of early lesions (postoperative endoscopic recurrence)[20]. *E. coli* was abnormally predominant (between 50 and 100% of the total number of aerobes and anaerobes) in early and chronic ileal lesions of CD patients[20]. Moreover, in any given patient, healthy and ulcerated mucosa are colonized by *E. coli* strains having the same ribotype profile, which is indicative of uniform colonization, regardless of the inflammatory state of the mucosa[35].

Abnormal colonization of the ileal mucosa is due to increased expression of CEACAM6, a receptor for adherent–invasive *E. coli* (AIEC)[13]. These bacteria have been isolated from ileal lesions of CD patients, and express the type 1 pilus variant, as opposed to the type 1 pilus expressed by *E. coli* MG1655[36]. CD-associated AIEC strains adhere to the brush border of primary ileal enterocytes isolated from CD patients, but not from control patients without IBD. AIEC adhesion is dependent on type 1 pilus variant expression on the bacterial surface[36] and on abnormal CEACAM6 expression on ileal epithelial cells in CD patients[13]. The significantly increased ileal CEACAM6 expression in the uninvolved ileal mucosa of CD patients compared to that in controls without IBD, suggests that patients expressing a basal level of CEACAM6 are genetically predisposed to express that molecule. Additionally, CEACAM6 expression in cultured intestinal epithelial cells is increased after interferon (IFN)-γ or tumor necrosis factor (TNF)-α stimulation, and after infection with AIEC bacteria, which indicates that AIEC can promote its own colonization in CD patients[13]. Accordingly, in patients expressing a basal level of CEACAM6, the presence of AIEC bacteria and the secretion of IFN-γ and TNF-α lead to amplification of colonization and inflammation.

INVASIVES PROPERTIES OF *E. COLI* STRAINS ASSOCIATED WITH CD

Analysis of *E. coli* strains isolated from early or chronic ileal lesions of patients with CD has revealed the presence of true invasive pathogens, since CD-associated bacteria efficiently invade a wide range of human epithelial cell lines, including Hep-2 cells and the intestinal cell lines Intestine-407, Caco-2 and HCT-8[37]. Their uptake is dependent on functioning host-cell actin microfilaments and microtubules[37]. Electron microscopy of epithelial cells infected with CD-associated bacteria has revealed a macropinocytosis-like process of entry, characterized by elongation of the membrane extensions, which surround bacteria at the sites of contact between entering bacteria and epithelial cells. Inside the host cells, CD-associated bacteria survive and replicate in the cytoplasm after lysis of the endocytic vacuole. The invasive process of CD-associated bacteria is unique since it does not possess any of the known genetic invasive determinants described for enteroinvasive, enteropathogenic, and enterotoxigenic *E. coli*, and *Shigella* strains. The major virulence factors of CD-associated AIEC that play a role in their invasive ability are type 1 pili that induce membrane extensions[36], flagella that confer bacterial mobility and down-regulate the expression of type 1 pili[38], outer membrane vesicles that deliver bacterial effector molecules to host cells[39], and outer membrane protein C (OmpC), which regulates the expression of several virulence factors *via* the sigma(E) regulatory pathway[40]. Interestingly, among these virulence

factors, the outer membrane vesicles of *H pylori* and *Pseudomonas aeruginosa* have been reported to induce pro-inflammatory responses[41,42], and bacterial flagellin can interact with Toll-like receptor (TLR) 5 to activate an innate immune response.

The invasive ability of AIEC strains can allow bacteria to translocate across the human intestinal barrier and move into the deep tissues. Consequently, AIEC can interact with resident macrophages and continuously activate immune cells. In addition, patients with CD are more likely to be sensitive to AIEC infection. Indeed, the *NOD2* gene, located on chromosome 16q12, has been identified as the first susceptibility gene for CD[11,12]. NOD2-deficient mice show loss of protective immunity in response to bacterial muramyl dipeptide, and mice are susceptible to *Listeria* infection *via* the oral route[43]. The 3020insC mutant of NOD2 associated with CD has impaired function as a defensive factor against intracellular bacteria in intestinal epithelial cells[44]. Thus, patients carrying NOD2 mutations are unable to control bacterial infections. The mutated NOD2 receptor does not contribute to pro-inflammatory gene transcription in response to bacteria, which results in an inadequate innate response to bacterial invasion and enables bacteria to accumulate. Such a poor innate response can lead to the formation of granulomas and thus, to the activation and perpetuation of a deregulated secondary adaptive response.

AIEC SURVIVAL AND REPLICATION WITHIN MACROPHAGES AND GRANULOMA FORMATION

The search for infectious agents likely to cause CD has focused mainly on intracellular pathogens that have evolved to resist phagocytosis and to persist within macrophages, and which may be involved in chronic antigenic stimulation leading to T-cell and macrophage activation. AIEC strains isolated from CD patients are able to survive and replicate extensively within murine macrophages[45]. At 48 h post-infection, the number of intracellular AIEC bacteria can increase up to 74-fold compared to the initial infection. In contrast to its behavior within intestinal epithelial cells[37], CD-associated bacterial replication does not require bacterial escape into the cytoplasmic compartment[45]. Within J774-A1 macrophages, AIEC bacteria induce the formation of a single spacious vacuole by fusion of initial phagosomes. The behavior of the AIEC strains within macrophages is different from that of other invasive bacteria. In contrast to most invasive bacteria that induce death of infected macrophages[46], no necrosis or apoptosis of AIEC-infected J774-A1 macrophages is observed even after 24 h post-infection[45]. Moreover, in contrast to many pathogens that escape from the normal endocytic pathway, AIEC bacteria are taken up by macrophages within phagosomes, which mature without diverting from the classical endocytic pathway, and share features with phagolysosomes[47]. To survive and replicate in the harsh environment encountered inside these compartments, including acid pH and proteolytic activity of cathepsin D, AIEC have elaborate adaptation mechanisms, for which acidity constitutes a crucial signal, to activate the expression of virulence genes[48]. The major virulence factors of CD-associated AIEC that have a role in their ability to survive and replicate within macrophages are the *htrA* gene that encodes the stress protein HtrA, essential for intracellular replication within macrophages[48], and the *dsbA* gene that encodes the periplasmic oxidoreductase DsbA, essential for AIEC LF82 to survive within macrophages, irrespective of the loss of flagellum and type 1 pilus expression[49]. LF82-infected macrophages release large amounts of TNF-α[45]. This result is in accordance with the fact that several studies have shown that T helper (Th)1 cytokines, such as IFN-γ, TNF-α, and interleukin (IL)-12, are secreted in excess in CD whereas in UC, an atypical Th2 immune response with secretion of IL-4 or transforming growth factor (TGF)-β was observed[50]. Continuous macrophage activation and TNF-α release in CD patients may be due to the sustained multiplication of intracellular AIEC bacteria within phagosomes, and may be involved in the formation of granulomas. Granulomatous inflammation is a histological hallmark of CD and infection with some intracellular bacteria. *E. coli* DNA is present in 80% of microdissected granulomas in CD patients[51]. Granulomatous responses to *E. coli* have been reported in animals, such as granulomatous colitis of boxer dogs or Hjarre's disease in chickens and turkeys. *E. coli* strains were isolated from 100% of granulomas in boxer dogs with colitis[52], and these bacteria resembled CD-associated AIEC in phylogeny and virulence gene profile[53]. In Hjarre's disease, mucoid *E. coli* has been isolated from tuberculoid lesions of the cecum and liver of chickens and turkeys, while intramuscular inoculation of pure bacterial cultures or triturated diseased tissues reproduced the disease[54-56]. Using an *in vitro* model of human granuloma[57], CD-associated AIEC LF82 were reported to induce aggregation of infected macrophages, some of which fused to form multinucleated giant cells and subsequent recruitment of lymphocytes. Analysis of the cell aggregates indicated that they are very similar to the early stages of epithelioid granulomas[58].

PREVALENCE OF AIEC IN IBD

AIEC strains have been found to be highly associated with ileal mucosa in CD patients[56]. Such pathogenic strains were isolated from ileal specimens of 36.4% of CD patients *vs* 6% of controls. In colonic specimens, AIEC strains were found in 3.7% of CD patients, 0% of UC patients, and 1.9% of controls. These strains are preferentially found in early recurrent lesions after surgery, thus indicating their role in the initiation of inflammation, and not just as secondary invaders. Another study has shown that mucosa-associated *E. coli*, which accounted for 53% of isolates, were more common in CD (43%) than in non-inflamed control patients (17%), while intramucosal *E. coli* were found in 29% of CD patients *vs* 9% of controls[30]. These studies support a central role for mucosa-associated AIEC in the pathogenesis of CD[30,56], since the translocation of these pathogenic bacteria through the intestinal mucosa may be a crucial step in the propagation of the inflammatory process.

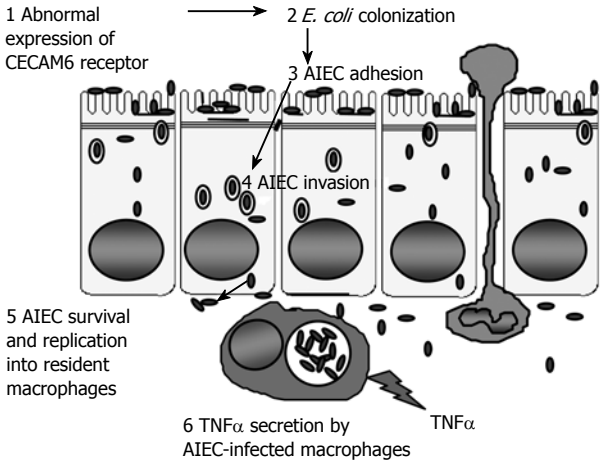

Figure 1 Sequential steps of the mechanisms of disease induced by AIEC bacteria: (1) abnormal expression of CEACAM6 in ileal mucosa of CD, inducing (2) AIEC colonization, (3) adhesion and (4) invasion, which allow the bacteria to cross the mucosal barrier. AIEC bacteria can (5) survive and replicate within infected macrophages in the lamina propria, and (6) induce TNF-α secretion.

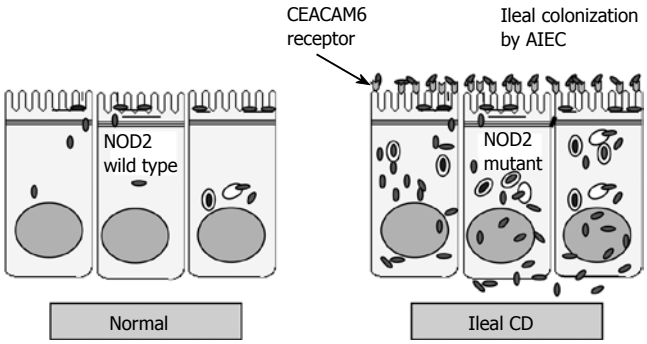

Figure 2 The infection cycle of AIEC may depend upon the ability of these pathogenic bacteria to colonize the gastrointestinal tract of genetically predisposed patients. Patients at high risk for developing severe ileal CD are those who overexpress CEACAM6 in the ileal mucosa, which allows AIEC colonization, and express the NOD2 3020insC mutant which has an impaired function as a defensive factor against intracellular bacteria in intestinal epithelial cells.

CONCLUSION

Various factors lend credence to the theory that AIEC is intimately linked to the etiopathogenesis of ileal CD. The high prevalence of AIEC in patients with ileal CD may be the first step in the establishment of a modified Koch's postulate that takes into account the genetic susceptibility of the host[30,56]. A possible role for AIEC in the etiopathogenesis of CD in susceptible hosts is summarized in Figure 1. The sequential steps involved in the induction of disease by the bacteria are: (1) abnormal colonization *via* binding to the CEACAM6 receptor, which is overexpressed in the ileal mucosa of CD patients[13]; (2) ability to adhere to and to invade intestinal epithelial cells, which allows bacteria to cross the mucosal barrier[37]; (3) survival and replication within infected macrophages in the lamina propria; and (4) induction of TNF-α secretion[45] and granuloma formation[58].

AIEC strains could colonize the ileal mucosa of CD patients by binding to CEACAM6, translocate across the human intestinal barrier to move into deep tissues, and once there, continuously activate immune cells. Patients having a high risk for developing severe ileal CD may be those who, in addition to expressing a variant of the NOD2 intracytoplasmic receptor[11,12], overexpress CEACAM6 at the surface of the ileal mucosa[13] (Figure 2). Host innate immune receptors that can be activated by AIEC components are mainly the transmembrane receptor TLR2 and the intracellular receptor NOD2. NOD2 is a negative regulator of the TLR2-mediated Th1 response, while the NOD2 3020insC mutation associated with CD is unable to inhibit TLR2 signaling, which skews the system toward an overactive Th1-mediated response[59]. This result provides a compelling explanation for why people carrying the NOD2 mutation might develop CD in response to abnormal colonization by AIEC[60]. The treatment of severe ileal CD could evolve from being almost exclusively surgical to management that places much greater emphasis on medical therapy, such as immunomodulators and anti-TNF-α agents, and also on antibiotic or probiotic treatments.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Innate immunity in inflammatory bowel disease

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Abstract

The human intestinal tract is home to an enormous bacterial flora. The host defense against microorganisms can be divided into innate and adaptive immunity. The former is the most immediate line of response to immunologic challenges presented by bacteria, viruses, and fungi. The mucosal immune system has evolved to balance the need to respond to pathogens while co-existing with commensal bacteria and food antigens. In inflammatory bowel disease (IBD), this hyporesponsiveness or tolerance breaks down and inflammation supervenes driven by the intestinal microbial flora. Bacteria contain compounds and are recognized by a variety of receptors, including Toll-like receptors (TLRs) and NODs (a family of intracellular bacterial sensors) and are potent stimuli of innate immune responses. Several mutations in these receptors have been associated with development of IBD.

Key words: Innate; Immunity; Toll-like receptors; Inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises two major forms of chronic inflammation of the intestine, Crohn's disease (CD) and ulcerative colitis (UC).

IBD is currently presumed to result from the complex effect of diverse genes conferring risk of disease and environmental factors, which when combined lead to an aberrant inflammation response. Recent evidence suggests that innate immune responses play an important role in initiating the inflammatory cascade and subsequent characteristic pathological adaptive immune responses[1]. The innate immune response is the first line of defense for microbial infections. In addition to genetic factors in IBD, numerous studies have implicated a key role of the intestinal microbiota in patients with IBD[2-4]. The contribution of luminal microbes to the pathogenesis of IBD is highlighted by reports that surgical diversion of the fecal stream effectively resolves CD inflammation distal to the surgical site[2].

The human intestinal tract mucosa is exposed to an enormous microbial flora. A single layer of epithelial cells separates the host tissues from luminal bacteria. Innate immune mechanisms are involved in this relationship, and likely contribute significantly to the protection of the host from invasion by luminal bacteria and provide a rapid response to pathogens. Immediate activation of innate immunity relies on the detection by the host of conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs), comprising diverse molecules from bacteria and viruses such as lipopolysaccharide, peptidoglycan, flagellin and lipoproteins[5].

Understanding of innate immunity has accelerated enormously with the discovery of many microbial sensors called "pattern recognition receptors" (PRRs). The toll-like receptor (TLR) and NOD receptor families of PRRs appear to play essential roles in mucosal homeostasis and alterations contribute to the pathogenesis of IBD.

THE TLR FAMILY

The mammalian TLR family consists of 13 mammalian members: each TLR having its intrinsic signaling pathway and inducing specific biological responses against microorganisms. Recognition of microbial components by TLRs triggers activation of signal transduction pathways, which then induce dendritic cell maturation and cytokine production, resulting in development of adaptive immunity[5].

Table 1 summarizes TLRs and the different molecular patterns associated with a broad range of microbes that they recognize. The activation of TLR signaling pathways originates from the cytoplasmic TIR domains. Four TIR domain-containing adaptors (MyD88, TIRAP/MAL, TRIF, and TRAM) play an important role in TLR signaling

Table 1 Molecular pattern recognition of NODs and TLRs

Receptor	Microbial motifs
NLR family	
NOD1	Lanthionine meso-diaminopimelic acid (meso-DAP) γ-D-Glu-meso-diaminopimelic acid (iE-DAP)
NOD2	Muramyldipeptide (MDP)
TLR family	
TLR1	Triacyl lipopeptides
TLR2	Lipoprotein, lipopeptides ($Pam_3CysSerLys_4$)
TLR3	dsRNA
TLR4	LPS
TLR5	Flagellin
TLR6	Diacyl lipopeptides
TLR7/TLR8	ssRNA
TLR9	Non-methylated CpG DNA
TLR11	Component of uropathogenic bacteria

pathway. These adaptors are associated with TLRs through homophilic interaction of TIR domains. Each TLR mediates distinctive responses in association with a different combination of these adapters[6].

THE NLR FAMILY

The mammalian NLR family comprises more than 20 members whose defining molecular characteristic is a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding NACHT domain and an N-terminal protein-protein interaction domain composed of a CARD (caspase activation and recruitment domain). Several studies have shown that several NLRs are necessary sensors of specific PAMPs.

The first NLRs reported to have a direct function as intracellular PRRs were NOD1 (CARD4) and NOD2 (CARD15). NOD2 detects muramyl dipeptide, the largest molecular motif common to Gram-negative and Gram-positive bacteria[7,8]. In contrast, NOD1 senses peptidoglycan containing meso-diaminopimelic acid (meso-DAP), which is more commonly found in Gram-negative bacteria[9,10].

NALP3 is a pyrin domain-containing NLR that activates the caspase-1 leading to interleukin 1β (IL-1β) and IL-18 processing, this protein is involved in sensing microbial components. In addition to NODs and NALP3 proteins, Ipaf (CARD12) and Nalp1 are intracellular sensors that detect intracellular flagellin, leading to inflammasome activation through a TLR5-independent pathway[11].

The signaling pathways downstream of NLRs include the NF-κB pathway, for NOD1 and NOD2, and activation of the caspase 1 inflammasome, for NAPLPs, Ipaf and Naip. NOD1 and NOD2 rapidly form oligomers and then transiently recruit receptor-interacting protein 2 (RIP2) through CARD-CARD interactions. The complex NOD-RIP2 then recruits the inhibitor of NF-κB kinase complex, which leads to activation of NF-κB. Several studies have shown that Ipaf and Naip can participate in the formation of inflammasomes (NALP1, NALP3 and Ipaf) that form in response to the detection of specific molecular motifs. It has been speculated that the formation of large protein complexes in a given inflammasome is sufficient to trigger caspase-1 activation[12].

NLRs are important in macrophage-mediated detection and control of bacterial infection *in vitro*. Ipaf is required for caspase-1 activation and IL-1β secretion in macrophages exposed to Gram-negative pathogen *Salmonella typhimurium*[13]. Cytosolic recognition of *Salmonella typhimurium* and *Legionella pneumophila* flagellins by Ipaf results in the induction of macrophage cell death and IL-1β secretion.

CONNECTION OF NOD AND TLR PATHWAYS

Intersection between TLR and NOD2 pathways is suggested by reports of synergistic induction of proinflammatory cytokines such as TNFα and IL-1β upon costimulation with MDP and specific TLR ligands[14,15]. MDP also substantially upregulated secretion of TNFα and IL-1β induced by ligands to five different TLRs ligands, TLRs 2, 4, 5, 7 and 9: ($Pam_3CysSerLys_4$, LPS, Flagellin, MALP-2 and R-848, respectively). Of note, these effects were observed in the presence of the most common NOD2 mutants associated with CD. In studies using mice lacking NOD2, Watanabe *et al* observed reduced responses to MDP, but enhanced responses to the TLR2 ligand peptidoglycan e.g., increases in IL-12. They interpreted these findings to suggest that the NOD2 signaling pathways normally down-regulate the TLR2 pathways. In their model, loss of function mutation of NOD2 together with TLR2 signals delivered by other bacterial products could result in enhanced cytokine responses to commensal bacteria by macrophages[16]. These findings suggest that interaction between NOD2 and specific TLR pathways may represent an important modulatory mechanism of innate immune responses, which is altered in some patients with CD.

TLRS IN IBD

TLRs are abundantly expressed on the surface of monocytes, macrophages, dendritic and epithelial cells. Alterations of TLR3 and TLR4 expression by intestinal epithelial cells have been described in IBD[17], suggesting that there is differential expression of TLR family members. Thus, primary intestinal epithelial cells of normal, non-diseased mucosa constitutively express TLR3 and TLR5, whereas TLR2 and TLR4 are present in much lower amounts. In active IBD, the expression of TLR3 and TLR4 was differentially modulated in the intestinal epithelium. TLR3 was significantly down-regulated in active CD but not in UC. In contrast, TLR4 was strongly up-regulated in both UC and CD. TLR2 and TLR5 expression remained unchanged in IBD.

Two common polymorphisms of TLR4 (Asp299Gly and Thr399Ile) have been described in humans. Asp299Gly has been associated with reduced responsiveness following lipopolysaccharide stimulation[18]. These polymorphisms have been associated with the development of CD and UC in Caucasian populations[19-21].

Recently, Pierik *et al*[22] showed that TLR1 R80T and TLR2 R753G polymorphisms were associated with pancolitis in UC patients, while a negative association

was observed between TLR6 S249P and proctitis in patients with UC. These results suggest that TLR2 and its co-receptors TLR1 and TLR6 are involved in the initial immune response to bacteria in the pathogenesis of IBD.

An important immune stimulatory effect mediated by the TLR family (TLR9) is induced by non-methylated CpG motifs found in bacterial DNA. In animal models of colitis, administration of CpG was able to ameliorate disease activity[23].

NODS IN IBD

Specific mutations of the NOD2 gene have been definitively associated with increased susceptibility to ileal Crohn's disease in Western (but not Asian) populations: Arg702Trp, Gly908Arg, and leu1007fsinsC (a frameshift mutation that truncates the carboxy terminal 33 amino acids)[24,25]. Heterozygous carriage of the risk alleles confers a 2-4 fold increased risk, and homozygotes or compound heterozygotes have a 20-40 fold increased risk[26]. More than 90% of all CD associated mutations are located in the LRR domain, suggesting that these may affect the function of NOD2 with respect to bacterial recognition and signaling. Transient transfection experiments indicate that CD-associated NOD2 mutants no longer activate NF-κB in response to MDP[27,28], which suggests that defective NF-κB activation facilitates infection of the lamina propria by enteric bacteria.

NOD2 mutants produce selective functional defects in leukocytes of patients with CD as shown by van Heel *et al*[8] who analyzed cytokine expression of peripheral blood mononuclear cells after exposure to MDP. In PBMC from CD patients the NOD2 ligand induced little TNFα and IL-1β, but strong IL-8 secretion. Furthermore, monocytes isolated from CD patients carrying the 1007fs (3020insC) mutation were reported to exhibit defects in the production of the proinflammatory cytokines, TNFα, IL-6 and IL-8, as well as the anti-inflammatory cytokine IL-10[29]. Dendritic cells derived from CD patients homozygous for leu1007fsinsC also fail to up-regulate the costimulatory molecules CD80 and CD86 in response to MDP and lack production of cytokines such as TNF-α, IL-12 and IL-10[30].

Evidence that NOD2 functions as an antibacterial factor in intestinal epithelial cells was demonstrated in Caco-2 cells stably expressing wild type NOD2 when infected with *Salmonella typhimurium*. This protective effect was absent in cells expressing a most common mutant NOD2 associated with CD (3020insC)[31].

NOD2 mutations in CD patients are also associated with diminished mucosal α-defensin expression[32]. Decreased β-defensin 1 and the lack of induction of both inducible antimicrobial peptides β-defensins 2 and 3 in CD could result in enhanced bacterial invasion and perhaps survival[33].

A study of 556 patients with IBD (294 CD and 252 UC) reported an association between the variant (rs695857) in nucleotides 30, 258 and 950 of the *NOD1* gene and the development of IBD. Another variant known as rs2907748 in nucleotides 30, 246 and 263 was also associated with the presence of UC and CD, particularly early onset of the disease (< 25 years)[34].

CROHN´S DISEASE

Marks *et al*[35] have provided provocative evidence that CD patients possess a generalized impaired innate immune response as reflected by diminished response to intradermal injection of killed bacteria as well as trauma of the skin or the intestine. When killed bacteria were injected into the forearms of CD patients, there was less blood flow to the injection site than non-CD patients. They also found that CD patients had reduced neutrophil accumulation and interleukin-8 (IL-8) production at sites of tissue trauma in the intestine and skin, although these findings need corroboration. This study supports the idea that CD may in some way be associated with relative inability to mount an acute inflammatory response compared to normal individuals.

Recent studies have suggested that the CD-associated NOD2 mutants might confer a milder defect in innate immune response than well-described innate immune deficiencies such as chronic granulomatous disease[8]. Rather than suppressing the secondary T-cell response, a different approach aims to normalize innate immune function through qualitative augmentation of neutrophil, macrophage and dendritic cell function. Clinical trials of granulocyte colony stimulating factor (G-CSF, specifically filgrastim) and GM-CSF (sargramostim) have suggested a benefit, although definitive evidence is not yet available[36,37]. However, G-CSF has a more limited effect on the innate immune system, acting primarily on neutrophils. GM-CSF is more widely and potently active, targeting a variety of cell types including not only neutrophils and monocytes as effector cells, but also intestinal epithelial cells that have receptors for GM-CSF.

CONCLUSION

The innate immune system is the first line of defense and provides a rapid response to pathogens. Elicitation of an innate immune response to bacterial products is mediated through families of pattern recognition receptors including the cell surface TLRs and cytosolic NODs that mediate the activation of NF-κB. Some mutations, TLRs, and NODs produce defects in sensing of pathogens and predispose the host to recurrent infections as well as perpetuation of chronic intestinal inflammation.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD), the most important being Crohn's disease and ulcerative colitis, results from chronic dysregulation of the mucosal immune system in the gastrointestinal tract. Although the pathogenesis of IBD remains unclear, it is widely accepted that genetic, environmental, and immunological factors are involved. Recent studies suggest that intestinal epithelial defenses are important to prevent inflammation by protecting against microbial pathogens and oxidative stresses. To investigate the etiology of IBD, animal models of experimental colitis have been developed and are frequently used to evaluate new anti-inflammatory treatments for IBD. Several models of experimental colitis that demonstrate various pathophysiological aspects of the human disease have been described. In this manuscript, we review the characteristic features of IBD through a discussion of the various chemically induced experimental models of colitis (e.g., dextran sodium sulfate-, 2,4,6-trinitrobenzene sulfonic acid-, oxazolone-, acetic acid-, and indomethacin-induced models). We also summarize some regulatory and pathogenic factors demonstrated by these models that can, hopefully, be exploited to develop future therapeutic strategies against IBD.

Key words: Inflammatory bowel disease; Experimental colitis; Dextran sodium sulfate; Trinitrobenzene sulfonic acid; Oxazolone; Pathogenesis

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INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), represents a chronic, relapsing and remitting inflammatory condition that affects individuals throughout life[1]. No completely effective therapeutic strategy has been established because the etiology of IBD remains largely unknown, although there has been extensive research on its pathogenesis. However, recent advances in the understanding of the pathophysiology of IBD have provided some clues for developing potentially helpful therapeutic tools.

Within the past two decades, several models of experimental colitis have been reported that demonstrate various pathophysiological aspects of human IBD. While no model serves as a complete surrogate for the human disease, some characteristically pathological features are open for investigation, depending on the method used to induce the experimental colitis. Experimental models of colitis enable us to dissect the pathogenic components during different phases of colitis, including acute, recovery and chronic phases. They also enable us to identify some pivotal immunological processes, as well as novel genes that are intimately involved in disease susceptibility.

In this review, we mainly focus on the role of functionally distinct factors, including immune cells, cytokines/chemokines, receptors/ligands, transcriptional factors, and enzymes/hormones, which maintain the homeostatic balance in the colon during the development of acute and chronic inflammation.

DSS-INDUCED COLITIS

The dextran sodium sulfate (DSS) model, originally reported by Okayasu *et al*[2] has been used to investigate the role of leukocytes in the development of colitis. Oral administration of 5% DSS in drinking water can induce not only acute, but also chronic colitis. One cycle of 3%-5% DSS administration for 5-7 d, followed by

regular water, results in extensive injury with complete crypt depletion (mainly basal crypt) and relatively slow regeneration of colonic epithelium. This regeneration is much slower than in other acute injury models, which use toxic substances such as acetic acid and ethanol[3]. The clinical features of this model include weight loss, loose stools/diarrhea, and rectal bleeding. Histopathological analysis typically reveals extensive crypt and epithelial cell damage, significant infiltration of granulocytes and mononuclear immune cells, and tissue edema, often accompanied with severe ulceration. In fact, because of the massive edema and subsequent ulceration during the acute phase, some researchers have wrongly used the DSS-induced colitis model by interpreting it as a model for human UC; however, this colitis is a simple model of acute chemical injury rather than chronic inflammation. Pathological scoring is generally performed on the distal segment of the colon, which is the most severely affected portion[3]. Histopathology, by hematoxylin and eosin staining, is scored based on three parameters: severity of inflammation (none, mild, moderate, severe), extent of inflammation (none, mucosa, mucosa and submucosa, transmural), and crypt damage (none, basal one-third damaged, basal two-thirds damaged, crypt lost but surface epithelium present, crypt and surface epithelium lost). It is noteworthy that long-term DSS administration produces colorectal carcinoma, which is similar to the dysplasia-carcinoma sequence seen in the course of cancer development in human UC[4].

Acute mucosal damage can be observed in both wild-type and severely combined immunodeficiency (scid) mice, which indicates that acquired immune responses are not involved in the induction of DSS-induced colitis[5]. The lesions observed in scid mice have been associated with increased production of macrophage-derived proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. While the role of luminal bacteria in the pathogenesis of DSS-induced colitis is unclear, this colitis can be ameliorated by treatment with antibiotics that are clinically effective in patients with IBD[1], which suggests the importance of commensal bacteria in the development of colitis[6]. Although the earliest change of acute DSS-induced colitis is a progressive disruption of colonic crypts during the chronic phase (14 d after stopping DSS), macrophages and $CD4^+$ T cells are more prominent in areas of wound healing in the basal portions of the lamina propria (LP). These $CD4^+$ T cells secrete increased levels of interferon (IFN)-γ and IL-4, which suggests that chronic immune activation mediated by both Th1 and Th2 cells play a pathogenic role in chronic DSS-induced colitis[7].

2,4,6-TRINITROBENZE SULFONIC ACID (TNBS)-INDUCED COLITIS

In 1995, Neurath *et al* described a novel murine model of intestinal inflammation induced by intrarectal administration of hapten reagent TNBS in ethanol solution. Simultaneous administration of TNBS and ethanol is required to induce TNBS colitis, because ethanol disrupts the epithelial layer and exposes the underlying LP to bacterial components. Intestinal inflammation induced by intrarectal administration of TNBS has many of the characteristic features of CD in humans, including severe transmural inflammation associated with diarrhea, rectal prolapse, weight loss, and induction of an IL-12-driven inflammation with a massive Th1-mediated response[8]. Interestingly, prior oral administration of TNBS in the form of trinitrophenol-haptenated colonic protein (TNP-CP) prevents colitis induced by intrarectal administration of TNBS[9,10]. The preventive effect is due to the induction in the LP of regulatory cells consisting of $CD4^+$ T cells that produce transforming growth factor (TGF)-β after oral administration of TNP-CP[10].

The susceptibility to TNBS colitis varies between different mouse strains; SJL and BALB/c are susceptible, whereas C57Bl/6 and 10 mice are resistant. The susceptibility has been shown to be related to a genetically determined high IL-12 response to the lipopolysaccharide (LPS) locus on chromosome 11 in SJL/J mice[11]. In a recent study, te Velde and colleagues compared gene expression profiles in the colons of three different models of colitis (DSS, TNBS and $CD45RB^{high}$ T-cell transfer models)[12]. As a result, a restricted number of genes were either up- or down-regulated in the TNBS colitis (21 genes) model compared to DSS-induced colitis (387 genes) and $CD45RB^{high}$ transfer model (582 genes)[12]. Of the 32 genes known to change transcriptional activity in IBD (*TNF, IFN-γ, Lt*β*, IL-6, IL-16, IL-18R1, IL-22, CCR2* and *7, CCL2, 3, 4, 5, 7, 11, 17* and *20, CXCR3, CXCL1, 5* and *10, Mmp3, 7, 9* and *14, Timp1, Reg3*γ*, Pap, S-100a8, S-100a9, Abcb1,* and *Ptgs2*), two (*Mmp14* and *Timp1*) are up-regulated in TNBS, 15 (*IL-6, IL-16, IL-22, CCL2, 3* and *11, CXCL1* and *5, Mmp3* and *14, Timp1, Reg3*γ*, Pap, S-100a9,* and *Ptgs2*) are up- or down-regulated in DSS, and 30 (except for *CCL11 and Timp1*) are up- or down-regulated in the CD45RB transfer colitis models. The study suggests that the pattern of gene expression in these colitis models closely reflects altered gene expression in human IBD[12].

OXAZOLONE COLITIS

In contrast to TNBS, which leads to colitis driven by a Th1-polarized type of T-cell response, administration of another haptenating agent, oxazolone, leads to a colitis associated with a Th2-polarized type of response. This model is induced by the rectal administration of oxazolone suspended in an ethanol vehicle. Although the SJL/J strain of mice was utilized in the original description[13], over half of the later studies have been performed using the C57Bl/6 strain. The induction of colitis in the C57 strain requires a presensitizing treatment, since this strain is resistant to haptenating agents[14]. For presensitization, 4.5 mg - 6 mg of oxazolone in 100% ethanol is injected into the abdominal wall of mice, followed by intrarectal administration of various doses of oxazolone in 50% ethanol after 5 d.

Oxazolone colitis is limited to the distal part of the colon, in contrast to TNBS colitis that is characterized as pan-colitic. Microscopically, the inflammation of oxazolone colitis manifests as relatively superficial ulceration[13]. An IL-4-driven Th2-type of response is predominant and is

Table 1 Pathogenesis of IBD models in DSS colitis

Pathogenic factors	
Categories	Factors (References)
Chemokines/cytokines	Migration inhibitory factor[114], LIX[115], L-18[33], CCR5[116], IL-1[117]
Adhesion molecules	CD98[118], β2 integrins (CD18/11a)[83], Integrin α1β1[81], VCAM-1[119]
Transcriptional factors	STAT3[74]
Toll like receptors and ligands	CpG motifs[92], Flagellin/TLR5[90]
Enzymes	Chitinase 3-like-1[102], Carbonic anhydrase Ⅳ[100], Eosinophil peroxidase[120], Caspase-1[105]
Hormones	Adiponectin[112], Resistin-like molecule β[121], Leptin[113], Osteopontin[122], Activins[123]
Others	Galanin-1 receptor[124]
Regulatory factors	
Categories	Factors (References)
T cells	γδT cells[23,24]
Cytokines/chemokines	BFGF[51], FGF2[125], TGF-α[46], TFF2[53], ITF[54], HGF[47,49]
Transcription factors	SOCS3[74], Nrf2[126], PPARγ[76,77], PPARδ[76]
Adhesion molecules	B2 integrins (CD11β)[83]
Receptors	TLR4[87], PG receptor EP-4[95], Pregnane X Receptor[127]
Enzymes	COX-2[94,96], COX-1[94], Matrix metalloproteinase-2[128]
Hormones	Estrogen[129], Growth hormone[130], Adiponectin[110]
Neuronal factors	Vagus nerve[131], IRE1β[132], Neurotensin[133]
Lipid-associated molecules	Lipoxin A4[134], Apolipoprotein A-Ⅳ[135]
Others	Dietary glycine[136], Follistatin[123], Bacterial superantigens[137], Thioredoxin-1[138]

characterized by increased IL-4/IL-5, but normal IFN-γ production. The inflammation is prevented by the systemic co-administration of intraperitoneal anti-IL-4 antibody. The proinflammatory Th2-dominant cytokine response is regulated by TGF-β, which limits both the extent and duration of the disease. The histological features and inflammatory distribution of oxazolone colitis resemble human UC[13].

OTHER CHEMICALLY-INDUCED COLITIS MODELS

In a search for novel experimental models of acute IBD, MacPherson and colleagues have found that intrarectal administration of 3%-5% acetic acid induces acute colitis in the distal part of the colon in rats[15]. The initial injury consists of epithelial necrosis and edema that variably extends into the LP, submucosa, or external muscle layers. Epithelial injury is mainly caused by organic acids specifically because hydroxyl chloride (pH 2.3) does not generally induce acute colitis[4]. In mice, administration of acetic acid within 4 h results in colonic epithelial destruction without inflammation, which is then followed by an influx of acute inflammatory cells, and reaches its maximum intensity at 12 h. The inflammatory response is caused by non-specific factors after disruption of the epithelial barrier. The chemical injury heals within days in mice or 2-3 wk in rats[16].

Whereas acetic acid produces acute inflammation restricted to the colon, another pro-inflammatory agent, indomethacin, has been used to induce acute ileitis. Fasted rats are treated subcutaneously with indomethacin 7.5 mg/kg in sterile sodium bicarbonate, which leads to an acute inflammatory response characterized by multiple deep, longitudinal ulcers in the distal jejunum and proximal ileum. This acute response reaches its maximum intensity at 24 h and is completely resolved within 7 d, whereas two daily subcutaneous injections of indomethacin produce a chronic inflammation that lasts at least 2 wk[17]. Luminal bacteria and their products significantly contribute to the exacerbation and perpetuation of the chronic phase of indomethacin-induced inflammation.

These models have the advantage of being easy to initiate and therefore would be useful in the initial screening of new drugs for acute epithelial injury. However, the injury in the first 24 h is nonimmunologic and thus is not suitable for drug therapy trials for human IBD.

FACTORS INVOLVED IN THE PATHOGENESIS OF THE MAIN CHEMICALLY INDUCED COLITIS MODELS

In the following section, we focus more on the factors involved in the fine balance between pathogenic and regulatory factors in the pathogenesis of DSS- (Table 1), TNBS- (Table 2), and oxazolone- (Table 3) induced colitis.

T cells

$CD4^+$ T cells play a key role in the development of most T-cell-mediated IBD models. For example, the increased production of IFN-γ, mainly produced by $CD4^+$ T cells, is detected in most models of Th1-mediated colitis[18]. By contrast, IL-4 and IL-13, produced by natural killer (NK) T cells, have been shown to play a key role in the pathogenesis of Th2-mediated colitis, including oxazolone-induced colitis[19]. NK1.1 positive lymphocytes are also essential for alleviation of TNBS-induced colitis in the presence of peripheral tolerance[20].

Although $CD8^+$ T cells represent a major T-cell subset, there is little information available regarding the role of $CD8^+$ T cells in the pathogenesis of colitis. $CD8^+$ T cell receptor (TCR)-positive $V\beta14^+$ T cells, which are increased in the LP and have a cytotoxic effect[21], have a pathogenic role in the development of TNBS-induced colitis.

By contrast, TCRγδ T cells are an evolutionarily

Table 2 Pathogenesis of IBD models in TNBS colitis

Pathogenic factors	
Categories	Factors (References)
T cells	Th1[8], $CD8^+$ TCR $V\beta14^+$ T cell[21], CEACAM1[27]
Cytokines/chemokines	IL-12[8,30], IFN-γ[8,34], IL-18[31,32,139], IL-6[73], IL-16[140], IL-17[38], TNF-α[29,141], MIP-α[142], MIP-3α[143]
Receptors	CD40[57,58], CD44v7[144], FcεRI[145], GITR[63,64], Complement receptor 3[146]
Transcription factors	NF-κB p65[65,67,147], RICK[69,70], MAPK p38[70], Smad7[72], Smad3[148]
Adhesion molecules	Integrinα1β1[80]
Enzymes	Poly (ADP-ribose) synthetase[149,150], Inducible nitric oxide synthase[151], Angiotensinogen[152], Vanin-1[107]
Hormones	Leptin[113], Ghrelin[153], Adiponectin[112]
Others	Geneticfactors[11], Glycolipid[154]
Regulatory factors	
Categories	Factors (References)
T cells	TCRγδ[25,26], NK1.1[20,155,156]
Cytokines/chemokines	TGF-β[10,44,45], IL-10[44,157,158], IL12 p40[34], IL12 p40-IgG2b[159], IL-2-IgG2b[160], IL-23[39], HGF[48], BFGF[51]
Receptors	PAR-2[61], TNFR1[56]
Transcription factors	STAT5b[161], Interferon regulatory factor-1[162], PPARγ[75]
Enzymes	Indoleamine 2, 3-dioxygenase[163]
Hormones	Adrenocortical hormones[164,165], NCX-101[166]
Neurotransmitters	Vasoactive intestinal peptide[167,168], μopioid receptor[169]
Lipid mediators	Lipoxin A4[170], Marine[171]
Bacteria and parasite related factors	Yersinia pseudotuberculosis[172], Lactic acid bacteria[173,174], Schistosome eggs[175], Cholera toxin subunit B[176,177]
Others	Galectin-1[178], Curcumin[179], Catalposide[180], Follistatin[123], Phex gene[181], FTY720[182], Matrine[183]

Table 3 Pathogenesis of IBD models in oxazolone colitis

Pathogenic factors	
Categories	Factors (References)
T cells	NKT[19], CEACAM1[27], Major basic protein[184], MHC class Ⅱ transactivator[185]
Cytokines/chemokines	IL-4[13], IL-13[19,40], EBI3[42]
Transcription factors	Smad7[72], NF-κB[67,68]
Others	Glycolipid[154]
Regulatory factors	
Categories	Factors (References)
T cells	Regulatory T cells[28]
Cytokines/chemokines	TGF-β[13]
Receptors	PAR-1[60]
Others	Budesonide[186]

conserved minor T-cell subset with characteristic properties that help maintain the homeostasis of epithelial cells, by providing a barrier between the luminal bacterial contents and underlying immune cells[22]. A regulatory role has been shown for TCRγδ T cells in DSS-[23,24] and TNBS-induced colitis models[25,26].

In addition to these populations, carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1; also known as CD66a) is a cell surface molecule that has been proposed to negatively regulate T cell function, and is associated with the regulation of T-bet-mediated Th1 cytokine signaling in TNBS- and oxazolone-induced colitis models[27].

Finally, regulatory T cells express the antigen non-specific suppressor factors transforming growth factor-β (TGF-β) and IL-10. Boirivant *et al* have shown that TNP-CP feeding cross-protects mice from an inflammatory response to a different hapten, oxazolone. This protective effect is associated with the appearance of mononuclear cells that produce regulatory cytokines[28]. This phenomenon of cross-protection could be exploited in designing novel treatments for IBD, because it demonstrates that an orally-administered antigen can induce production of regulatory cells that are able to suppress inflammation induced by a different type of antigen.

Cytokines/chemokines/growth factors

TNBS injection results in a transmural infiltrative colitis associated with an IL-12-mediated Th1-immune response[8]. In most cases, a single dose of TNBS is administered at the starting point of the experiment. In subsequent studies of IL-12, it has been reported that mucosal TNF-α is necessary for the initiation and perpetuation of TNBS colitis, since TNF-α-deficient mice are resistant to TNBS, and the colitis is extremely severe in mice that over-express TNF-α[29]. This result suggests that TNFα acts as a proximal co-factor for IL-12 or IL-18 production. One possible mechanism of amelioration by anti-IL-12 antibody treatment is through the induction of Fas-mediated apoptosis of Th1 cells[30].

Watanabe and colleagues have shown that TNBS-induced colitis is mediated by macrophage-derived IL-18[31]. In fact, neutralization with anti-IL-18 antibody results in dramatic attenuation of mucosal inflammation, and the administration of TNBS fails to induce significant colitis in IL-18 knockout (KO) mice. These results have been confirmed by another group who have demonstrated that recombinant human IL-18 binding protein isoform (rhIL-18BPa) leads to a significant reduction in TNBS-induced colitis, by decreasing local TNF-α production[32]. Interestingly, IL-18 is also a primary mediator of the inflammation in DSS-induced colitis, while neutralization of IL-18 attenuates intestinal damage in that colitis model[33].

In Th1-mediated colitis, the use of agents that block IL-12 secretion or activity provides the most direct approach for attenuating inflammation because IL-12 is critical for regulation of differentiation and activation of Th1 cells[8,30]. It has been demonstrated that IL-12p40 KO mice develop severe TNBS-induced colitis. Moreover, administration of IL-12p40 neutralizing antibody increases pathology in IL-12p35 KO mice, which suggests that IL-12p40, in contrast

to IL-12p70, exerts the major regulatory function in TNBS-induced colitis[34]. However, IL-12p40 forms heterodimers, not only with IL-12p35 (IL12p35p40; IL-12p70), but also with IL-23p19 (IL-23p19p40); a finding that raises the possibility that activity previously ascribed to IL-12 may be attributable to IL-23. Recently, it has been revealed that IL-23 is a key effector cytokine in the immune system of the intestine[35]. IL-23 specifically expands a pathogenic population of $CD4^+$ T cells called Th-17 cells, which produce IL-17A, IL-17F, IL-6 and TNF-α[36,37]. Indeed, IL-17R KO mice are protected against TNBS-induced colitis[38]. By contrast, Becker *et al*[39] have reported that IL-23 cross-regulates IL-12 production in T-cell-mediated TNBS colitis, since mice lacking the p19 subunit of IL-23 are highly susceptible to TNBS-induced colitis, and inhibition of IL-12p40 rescues IL-23p19 KO mice from lethal disease. These discrepancies regarding the role of IL-23 may result from different experimental models; therefore, further characterization should help in developing new therapeutic treatments for patients.

As for the Th2-type responses, oxazolone colitis is associated with increased production of IL-4/IL-5, and is prevented by the systemic co-administration of anti-IL-4 antibody[13]. Heller *et al*[19] have shown that IL-13, mainly produced by NK T cells, is a significant pathogenic factor in this model, since its neutralization by the decoy receptor IL-13Rα2-Fc prevents disease. As well, IL-13 induces TGF-β1, generally considered to be an anti-inflammatory cytokine, through IL-13Rα2 in oxazolone-induced colitis, and prevention of IL-13Rα2 expression leads to the marked down-regulation of TGF-β1 production and collagen deposition in bleomycin-induced lung fibrosis, during prolonged inflammation[40].

As an IL-12p40-related protein, it has been reported that Epstein-Barr virus-induced gene 3 (EBI3) dimerizes with a novel p28 subunit (which has homology to IL-12p35) to form the cytokine IL-27[41]. IL-27 has been shown to function as a proliferation factor for naïve, but not memory, $CD4^+$ T cells, and to synergize with IL-12 to stimulate IFN-γ production[41]. That EBI3 KO mice have been found to be resistant to oxazolone-induced colitis suggests that this molecule plays a crucial role in the induction of Th2-type immune responses[42].

Several families of growth factors regulate a wide spectrum of processes integral to IBD; including protection of the intestinal mucosa and activation, as well as regulation of the intestinal immune system. These factors mediate mucosal repair, restitution, remodeling and resolution of inflammation following tissue damage[43]. It is now widely accepted that TGF-β has an important function in regulating inflammation and tissue repair. Fuss *et al*[44] have elegantly demonstrated the relationship between TGF-β and IL-10 in the regulation of Th1-mediated inflammation in TNBS-induced colitis, by performing a study in which mice were fed a haptenated colonic protein and then administered either anti-TGF-β or anti-IL-10 antibody, at the time of subsequent rectal administration of TNBS. Anti-TGF-β antibody administration prevents TGF-β secretion, but leaves IL-10 secretion intact, whereas anti-IL-10 antibody administration inhibits both TGF-β and IL-10 secretion. Their data suggest that TGF-β alone is the primary mediator of counter-regulatory Th1-type mucosal inflammation, and that IL-10 is necessary as a secondary factor that facilitates TGF-β production, but does not act as a suppressor cytokine by itself. Interestingly, Kitani *et al*[45] have shown that single intranasal administration of DNA encoding active TGF-β prevents the development of Th1-mediated TNBS colitis. This study shows that following treatment, TGF-β-producing T cells and macrophages are found in the LP and spleen, in which they hypothetically act to prevent induction of TNBS colitis. Therapeutic strategies involving TGF-β-encoding DNA may provide beneficial effects in treating intestinal inflammation.

The role of TGF-α in the small intestine and colon has not been studied as extensively as it has been in the gastric mucosa. In DSS colitis, TGF-α is a mediator of protection and/or healing in the colon, which is demonstrated by the absence of disease in TGF-α-KO mice[46].

Hepatocyte growth factor (HGF) may be a critical regulatory factor in IBD since HGF activator-KO mice are unable to survive after DSS or acetic acid-induced colitis[47]. HGF promotes migration of gastrointestinal epithelial cells and accelerates wound repair by mucosal cells. The importance of HGF has been confirmed by the intrarectal administration of HGF-expressing adenovirus in TNBS-treated mice, which leads to significant improvements in mucosal damage[48]. The same group has also demonstrated the therapeutic effects of naked gene therapy of HGF in the DSS-induced colitis model[49]. Taking these results together, HGF gene delivery may be very useful as a therapeutic strategy for human IBD.

As well as HGF, basic fibroblast growth factor (bFGF or FGF-2) also improves mucosal damage by enhancing epithelial cell restitution and proliferation in the gastrointestinal tract[50]. In fact, rectal administration of human recombinant bFGF (hrbFGF) ameliorates DSS-induced colitis by significantly reducing the gene expression level of TNF-α[51]. Not only DSS-, but also TNBS-induced colitis is improved by the administration of hrbFGF, which not only enhances survival rate, but also up-regulates levels of cyclooxygenase (COX)-2, TGF-β, intestinal trefoil factor (ITF), and vascular endothelial growth factor (VEGF) in the colon[51].

Lastly, the trefoil factor family is comprised of three peptides; trefoil factor family 1 (TFF1), spasmolytic polypeptide (SP also known TFF2), and ITF (also known as TFF3). TFF2 is a low-molecular-weight protein that is up-regulated in gastric tissues infected with *Helicobacter* or affected by other inflammatory conditions[52]. TFF2 KO mice are susceptible to DSS-induced colitis, with prolonged colonic hemorrhage and persistent weight loss[53]. The importance of ITF in the modulation of inflammation, wound healing, and protection of the intestinal mucosa is supported by experiments in ITF KO mice, which have shown increased susceptibility and delayed wound healing during DSS- and acetic acid-induced colitis[54].

Receptors

TNF-α plays a central role in the pathology of Th1-mediated colitis such as CD; however, the role of its receptors, TNF receptor-type I (TNFR1) and -type Ⅱ

(TNFR2) in mediating pathology has not been fully explored. TNFR2 expression and signal transducer and activator of transcription (STAT) 3 activation in colonic epithelial cells (CECs) are markedly up-regulated during the recovery phase of DSS-induced acute colitis[55]. Recently, it has been reported that TNFR1 KO mice lose more weight and have increased mortality compared with wild-type mice, while TNFR2 KO mice lose less weight and have an improved survival rate compared to wild-type mice in TNBS-induced colitis. These results suggest that TNF-α signaling through TNFR1, but not TNFR2, is protective in mouse models of IBD[56].

As for Th1-type responses, CD40L-CD40 interaction is crucial for the priming of Th1 cells *via* the stimulation of IL-12 secretion by antigen-presenting cells (APC) in TNBS-induced colitis. The administration of anti-CD40L antibody prevents IFN-γ production and TNBS-induced colitis, which suggests that the Th1 response may be mediated by CD40L-CD40 interactions[57,58].

Recent studies have demonstrated that the proteinase-activated receptors (PARs), a family of G protein-coupled receptors activated by serine proteinases, have an important anti-inflammatory role in the colon. PAR-1 and -2 are highly expressed in CECs and neuronal elements, and are involved in regulating secretion by the epithelial cells of salivary glands, stomach, pancreas and the intestine[59]. Intracolonic administration of PAR-1 agonist in oxazolone-treated mice efficiently inhibits colitis[59]. By contrast, the inflammatory responses in PAR1 KO or PAR-1 antagonist-treated mice are exacerbated in oxazolone-induced colitis[60]. As well, PAR-2 activation prevents the development of TNBS-induced colitis[61].

Finally, the glucocorticoid-induced TNFR (GITR)-related gene is a member of the TNFR superfamily that is constitutively expressed at high levels on $CD4^+$ $CD25^+$ regulatory T cells, and at low levels on unstimulated T cells, B cells and macrophages[62]. GITR signalling in $CD4^+$ T cells is involved in the development and progression of colitis[63], while deletion of GITR protects against TNBS-induced colitis by reducing innate immune responses and effector T-cell activity[64].

Transcription factors

Nuclear factor (NF)-κB is the key transcription factor for pro-inflammatory responses, and is thought to be important in the initiation and progression of both human IBD and animal models of colitis[65,66]. Disease activity in mice with TNBS-induced colitis is inhibited by antisense oligonucleotides that inhibit the p65 subunit of NF-κB, which suggests a critical role for NF-κB in mediating inflammatory responses[65]. Attempts to control mucosal inflammation by the use of agents that block the NF-κB pathway have had some success in murine models. For example, it has been shown that administration of NF-κB decoy oligodeoxynucleotides (decoy ODNs) encapsulated in a viral envelope prevents the development of TNBS- and oxazolone-induced colitis by inhibiting production of IL-23/IL-17[67]. De Vry *et al* have used a chemically modified, non-viral NF-κB decoy and have shown that the NF-κB decoy ameliorates disease severity in TNBS-, DSS- and oxazolone- induced colitis. These studies suggest that NF-κB decoy ODNs are effective in attenuating Th1- as well as Th2-mediated colitis, and this would be a potentially useful therapeutic strategy for human IBD[68]. In addition to NF-κB, mitogen-activated protein kinase (MAPK) p38 is also a crucial mediator of inflammation. Inhibition of NF-κB and MAPK p38 by SB203580 is able to attenuate the inflammatory response in TNBS-induced colitis models[69,70].

By contrast, TGF-β1 functions as a negative regulator of T-cell immune responses, signaling target cells through the Smad family of proteins. Smad7, an inhibitor of TGF-β1 signaling, is over-expressed in the intestinal mucosa and purified mucosal T cells isolated from patients with IBD[71]. Oral administration of antisense oligonucleotide of Smad7 also ameliorates inflammation in TNBS- and oxazolone-induced colitis, by restoring TGF-β1 signaling *via* Smad3[72].

It has been demonstrated that cytokines exert their biological functions through Janus tyrosine kinases and STAT transcription factors. An experiment blocking the IL-6 receptor has demonstrated that IL-6 plays an important role in the development of Th1-mediated TNBS-induced colitis by activating the STAT3 signaling pathway[73]. Indeed, STAT3 was most strongly tyrosine-phosphorylated in human UC and CD patients and in DSS-induced colitis in mice[74]. These results suggest that the IL-6/STAT3 pathway plays a crucial role in the development and perpetuation of DSS-induced colitis.

Lastly, peroxisome proliferator-activated receptor γ (PPARγ) is a lipid-activated transcription factor, and PPARγ heterozygous mice are highly susceptible to TNBS-[75] and DSS-induced colitis[76]. It has also been reported that mice with a targeted disruption of PPARγ in macrophages display an increased susceptibility to DSS-induced colitis[77]. Therefore, activation of PPARγ may potentially protect against human IBD.

Adhesion molecules

Trafficking, activation and retention of leukocytes within inflamed tissues are mediated by several classes of specialized adhesion glycoproteins[78]. Collagens represent the most abundant extracellular matrix protein, and the major cell surface receptors for collagens are integrins[78,79]. The collagen-binding integrin α1β1 mediates inflammation in TNBS-[80] and DSS-induced colitis[81], which suggests the importance of α1β1-mediated adhesive leukocyte/matrix interactions in regulating mucosal inflammatory responses. Leukocyte β2 integrins are heterodimeric adhesion molecules consisting of a common β subunit (CD18) and different α subunits (CD11a-d)[82]. In DSS-induced colitis, leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18) seems to have a pathogenic role, whereas Integrin alpha M (Mac-1α, CD11b/CD18) serves in a regulatory capacity[83]. Much attention has been focused on the role of α4 integrin in IBD, but it has recently been reported that neutralization therapy may result in undesirable complications such as multifocal leukoencephalopathy[84].

Toll-like receptors (TLRs) and their ligands

It is widely suspected that IBD arises from a dysregulated mucosal immune response to luminal bacteria. TLRs,

which are pattern-recognition receptors expressed by both immune and non-immune cells, play a pivotal role in host/microbial interactions and have two distinct functions-protection from infection and control of tissue homeostasis, depending on the recognition of pathogens or commensals[85-88]. TLRs send intracellular signals in response to intestinal commensal or pathogenic microbes that contain or release conserved molecular patterns, such as LPS, bacterial lipoprotein, bacterial cytosine-guanosine dinucleotide (CpG) DNA, and bacterial flagellin. Activation of TLRs results in the activation of the innate and/or adaptive immune response[85]. In this context, TLRs play an important role in the maintenance of intestinal homeostasis. TLR4 recognizes LPS, and transduces a proinflammatory signal through the adapter molecule myeloid differentiation marker 88 (MyD88)[86]. DSS treatment of TLR4 KO and MyD88 KO mice has been shown to induce earlier and more severe colitis compared to that in wild-type mice, which suggests that TLR4 signaling through MyD88 is an important suppressor of the inflammatory response to chemical injury[87].

Bacterial flagellin specifically stimulates TLR5 and activates MAPK and NF-κB-related signaling pathways, which leads to the production of macrophage inflammatory protein 3α (MIP3α) and IL-8[89]. Flagellin exposure exacerbates inflammation in DSS-induced colitis, but not in the intact colon[88]. By contrast, a TLR2 specific agonist, peptidoglycan or lipoteichoic acid, does not cause any inflammatory response[90].

Lastly, TLR9 is critical for the recognition of the CpG motif of bacterial DNA[91]. DSS-induced colitis is less severe in TLR-9 KO mice[92], and treatment of mice with an adenovirus expressing CpG-ODN that is known to block CpG effects results in significant amelioration of DSS-induced colitis[92], which indicates that ODN inhibition of the immune-stimulating properties of bacterial DNA may offer a novel and specific tool for the treatment of IBD.

Enzymes

Although intestinal epithelial cells constitutively express COX-1, COX-2 is induced only during inflammatory conditions. Enzymatic activity of these COX isoforms produces prostaglandins (PGs) that have proinflammatory roles mediating fever, hyperalgesia, vascular permeability and edema. However, PGs also have a protective role against gastrointestinal injury[93]. The linkage between COX-2 and PGE2 for protection against colitis has been highlighted in various studies. For example, COX-2 KO mice are more susceptible to DSS-induced colitis, which correlates with their inability to produce PGE2[94]. Kabashima *et al*[95] have used mice deficient in prostaglandin receptor EP4 and examined the roles of prostanoids in DSS-induced colitis; their mice developed severe colitis, which suggests that EP4 maintains intestinal homeostasis by keeping mucosal integrity and down-regulating immune responses. It has also been shown that COX-2-derived PGE2 is important in TLR4-related mucosal repair[96], and that COX-2 has a protective effect against acetic-acid-induced colitis[97,98]. These results suggest that COX-2 has a pivotal role in the maintenance of mucosal homeostasis. However, there is controversy about whether COX-2 inhibitors worsen symptoms of human IBD[99].

Through the use of DNA microarray analysis, our group has demonstrated that several detoxification-associated molecules, which contribute to the prevention of inflammation by regulating physiological balance under normal conditions, are highly down-regulated in CECs in chronic colitis[100]. Among the up-regulated detoxification-associated molecules, carbonic anhydrase (CAR)-Ⅳ is an important enzyme involved in the suppression of acidification, by regulating mucosal bicarbonate concentration[101]. Unexpectedly, inhibition of CAR-Ⅳ suppresses the severity of DSS-induced colitis but enhances CEC proliferation, which raises the possibility that CAR-Ⅳ may have a pathogenic role under inflammatory conditions. Microarray analysis also identifies chitinase 3-like-1 (CHI3L1) as being specifically up-regulated in inflamed mucosa[102]. The expression of CHI3L1 protein is detectable in LP and CECs in several murine colitis models, and also in IBD patients, but is absent in normal controls. Anti-CHI3L1 antibody administration significantly ameliorates DSS-induced colitis, which suggests that inhibition of CHI3L1 activity may be a novel therapeutic approach for IBD. Our group is currently investigating this possibility by utilizing murine models of chronic colitis.

As well, IL-1β-converting enzyme (ICE), also known as caspase-1, is an intracellular protease that cleaves the precursors of IL-1β and IL-18 into active cytokines[103,104]. ICE deficiency results in protection from DSS-induced colitis, accompanied by the reduced release of the proinflammatory cytokines IL-18, IL-1β and IFN-γ[105].

Lastly, recent studies have identified Vanin-1 as being involved in the regulation of innate immunity. Vanin-1 is an epithelial ectoenzyme with pantetheinase activity, which is involved in the metabolic pathway of pantothenate (vitamin B5), and provides cysteamine to tissues[106]. Vanin-1 deficiency protects from TNBS-induced colitis. Additionally, by antagonizing PPARγ, Vanin-1 promotes the production of inflammatory mediators by intestinal epithelial cells[107]. This study suggests that Vanin-1 is an epithelial sensor of stress that exerts control over innate immune responses in tissues. As such, it has been proposed as a potential new therapeutic target for IBD.

Hormones

It has been demonstrated that adipose tissue secretes a variety of biologically active molecules[108]. Adiponectin (APN) is an adipose tissue-derived hormone and is considered to be a member of the expanding family of adipokines[109]. APN has a protective role against DSS-induced murine colitis, but not TNBS-induced disease[110], by inhibiting the production of chemokines such as monocyte chemoattractant protein-1 and MIP-2 in CECs, and the subsequent inflammatory response. However, a proinflammatory role for APN in synovial fibroblasts[111] and CECs[112] has recently been suggested. APN exerts proinflammatory activity in the colon by producing proinflammatory cytokines and inhibiting the bioactivity of protective growth factors such as bFGF and heparin-

binding epidermal growth factor. It is interesting to note that APN KO mice are highly protected from both DSS- and TNBS-induced colitis[112].

Finally, leptin, a regulator of food intake and energy expenditure, can also modulate immune and inflammatory responses. Leptin-deficient (ob/ob) mice exhibit less severe colitis compared to wild-type mice in DSS and TNBS models, while replacement of leptin in ob/ob mice converts disease resistance to susceptibility, which indicates that leptin deficiency accounts for the resistance to acute DSS- and TNBS-induced colitis[113]. It has also been shown that phosphorylation of STAT3 and induction of COX-2 are absent in the colon of ob/ob mice[113]. Therefore, leptin represents a functional link between the endocrine and immune systems.

CONCLUSION

Dysregulated immune responses initiated by microbial-host interactions contribute to the development and perpetuation of both murine colitis and, most likely, to human IBD. In this process, intestinal epithelial cells play important roles linking innate and acquired immune responses. In this review, we have focused primarily on the role of functionally distinct factors in the pathogenesis of chemically-induced models of intestinal inflammation during acute, recovery and chronic phases. The increasing clinical use of biological therapy in human IBD illustrates the potential benefits that may be derived from molecular analysis of immunopathogenesis. However, the long-term effects of such therapy have still not been determined, and concerns regarding potentially increased risks of infection or tumor development have been raised, given the essential roles of innate and acquired immunity in host defense. In this respect, topical treatment would have the advantage of selectively targeting local immune responses while sparing systemic immune protective mechanisms. Therefore, we need to find agents that have more targeted effects or take advantage of local delivery systems that target diseased lesions, such as is seen with oligonucleotide-based therapeutics. The different animal models provide an easy means to study factors involved in pathogenesis and to test new therapeutic agents for human IBD.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Genetic factors associated with the development of inflammatory bowel disease

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are complex polygenic disorders, characterized by several genes together with environmental factors contributing to the development of inflammatory bowel disease (IBD). Recent advances in research on genetic susceptibility have allowed the identification of diverse genes at different levels: (1) Innate immunity; (2) Antigen presentation molecules; (3) Epithelial integrity; (4) Drug transporter; (5) Cell adhesion. The application of genetic testing into clinical practice is close and all genetic markers may have several clinical implications: prediction of disease phenotype, molecular classification, prevention of complications, and prognosis.

Key words: Genetic; Susceptibility; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing inflammatory bowel diseases (IBD) of unknown etiology. CD and UC are complex polygenic disorders, characterized by several genes together with environmental factors contributing to the development of IBD.

A variety of epidemiological and clinical data suggest that genetic factors are intimately involved in the pathogenesis of IBD including familial aggregation pattern of disease with a much higher disease frequency in first degree relatives of affected individuals compared with the general population. Twin studies provide the argument for a genetic basis for IBD, with a much higher rate of disease concordance observed in monozygotic than in dizygotic twins and wide variations in the incidence and prevalence of IBD among different populations[1].

IBD is now considered a non-Mendelian polygenic disorder with important environmental interactions (e.g., microbial factors, smoking).

There are two main approaches to identifying genes in complex multifactorial diseases: the positional cloning approach based on linkage studies, and the candidate gene approach based on association studies. Linkage analysis studies the cosegregation of the disease with a marker within the families. Linkage analysis allows scanning of the whole genome. Eleven of these total genome scans have been undertaken in IBD, resulting in a number of susceptibility regions on chromosomes 1, 3, 4, 5, 6, 7, 10, 12, 14, 16, 19 and X[2]. According to their initial date of reporting and independent confirmations, the regions on chromosomes 16q, 12, 6, 14, 5, 19, 1, 16p and 10 have been renamed IBD 1 to IBD 9, respectively. However, new genes have been reported recently. All susceptibility genes discovered can be categorized into different levels of susceptibility: (1) Innate immunity; (2) Human leucocyte antigen (HLA) molecules; (3) Epithelial integrity (4); Drug transporter; and (5) Cell adhesion.

INNATE IMMUNITY

Understanding of innate immunity has progressed enormously with the discovery of many microbial sensors called pattern recognition receptors (PRRs). The toll-like receptor (TLR) and nucleotide oligomerization domain (NOD) receptor families of PRRs appear to play essential roles in mucosal homeostasis and their alterations contribute to the pathogenesis of IBD.

NOD2 is expressed constitutively in macrophages, neutrophils and dendritic cells[3], as well as in Paneth cells and is induced in epithelial cells[4]. NOD2 is a cytoplasmic protein that serves as a microbial sensor, and its leucine-rich repeat (LRR) domain is required for recognition of muramyl dipeptide (MDP), a fragment of peptidoglycan

present in bacterial cell walls. The ligand MDP ultimately leads to activation of the transcription factor nuclear factor kappa B (NF-κB), and induction of proinflammatory cytokines[5,6]. Specific mutations of the NOD2 gene have been definitively associated with increased susceptibility to ileal Crohn's disease in Western (but not Asian) populations: Arg702Trp, Gly908Arg, and leu1007fsinsC (a frame shift mutation that truncates the carboxy terminal 33 aminoacids)[7,8]. Heterozygous carriage of the risk alleles confers a 2-4 fold increased risk, and homozygotes or compound heterozygotes have a 20-40 fold increased risk[9]. More than 90% of all CD- associated mutations are located in the LRR domain, suggesting that these may affect the function of NOD2 with respect to bacterial recognition and signaling.

NOD1/ CARD4 (Caspase Recruitment Domain 4) plays a role in colonic epithelial defense against *E. coli* and *S. flexeneri* and mediates NF-κB activation[3,10]. Recently, genetic variants of NOD1 have been shown to be associated with disease susceptibility. In a recent study of 556 patients with IBD (294 CD and 252 UC), an association between the variant (rs695857) in nucleotides 30, 258 and 950 of NOD1 and the development of IBD was found. Another variant known as rs2907748 in nucleotides 30, 246 and 263 was also associated with the presence of UC and CD and even with the early onset of the disease (< 25 years of age)[11].

TLRs are abundantly expressed on the surface of monocytes, macrophages, dendritic and epithelial cells. Alterations of TLR3 and TLR4 expression by intestinal epithelial cells have been described in IBD[12], suggesting that there is differential expression of TLR family members. Two common polymorphisms of TLR4 (Asp299Gly and Thr399Ile) have been described in humans. Asp299Gly has been associated with reduced responsiveness following lipopolysaccharide stimulation[13]. These polymorphisms have been associated with the development of CD and UC in Caucasian populations[14-16]. Pierik *et al*[17] showed that TLR1 R80T and TLR2 R753G polymorphisms were associated with pancolitis in UC patients, while a negative association was observed between TLR6 S249P and proctitis in patients with UC. These results suggest that TLR2 and its co-receptors TLR1 and TLR6 are involved in the initial immune response to bacteria in the pathogenesis of IBD.

ANTIGEN PRESENTATION MOLECULES

The major histocompatibility complex (MHC) region is the region studied most extensively. Human leucocyte antigen (HLA) class Ⅱ molecules present partially digested antigen to the T-cell receptor and play a central role in the immune response. The mechanism by which classical HLA class Ⅱ genes exert their influence in IBD is unknown. Different HLA molecules may bind preferentially to different peptides, or bind the same peptide with varying affinity. In IBD, cross reactivity (known as molecular mimicry) may exist between the peptides derived from bacterial luminal flora and from self antigens present in the gut. This may lead to the generation of auto reactive T cells which contribute to disease pathogenesis. HLA-DRB1 is the most extensively studied gene in IBD. In a meta-analysis made by Stokkers *et al*[18], positive associations between UC and HLA-DR2, HLA-DRB1*1502 (OR = 3.74, CI: 2.2-6.38), HLA-DR9 (OR = 1.54, CI: 1.06-2.24) and HLA-DRB1*0103 (OR = 3.42, CI: 1.52-3.69) were found; a negative association was found with HLA-DR4 (OR = 0.54, CI: 0.43-0.68). Another study found that HLA-DRB1*0103 allele was associated with UC and its severe manifestations such as colectomy and pancolitis (P = 0.003, OR = 3.6, CI 95%: 1.46-8.9), while HLA-DRB1*15 allele was only associated with pancolitis in patients with UC (P = 0.001, OR = 8.5)[19].

On the other hand, HLA class Ⅲ genes have been associated with IBD. Several studies have shown the role of tumor necrosis factor α (TNF α) polymorphisms in IBD. There are specific genetic polymorphisms involving TNFα that influence the amount of cytokine produced. Bouma *et al*[20] reported an association between the polymorphism of *TNF*α gene promoter region at -308 position and UC, and this finding was confirmed by other studies[21, 22].

EPITHELIAL INTEGRITY

The organic cation transporter (OCTN) is a family of transporter proteins for organic cations, and may also transport carnitine, an essential cofactor of the metabolism of lipids. Carnitine is involved in the transport of long-chain fatty acids into the mitochondria. There is evidence that inhibition of fatty acid oxidation in the epithelium of the colonic mucosa is associated with the development of UC. There are two subtypes of this gene, OCTN1 and OCTN2, and some mutations have been reported in them: SLC22A4 1672C/T for OCTN1 and SLC22A5-207G/C for OCTN2, which are associated with the development of CD. The presence or combination of these mutations constitutes TC haplotype, which is associated with ileal, colonic and perianal affection and onset and the need of surgical treatment in CD[23, 24].

DLG5 (Drosophila long disc homologue 5) gene is a member of the membrane associated guanylate kinase gene family which encodes cell scaffolding proteins and seems to play a role in the maintenance of intestinal epithelial cells, and its mutations have been involved in a rise in intestinal permeability[25]. DLG5 is a widely expressed protein found in many tissues such as the placenta, small bowel, colon, heart, skeletal muscle, liver and pancreas. It is important in signal transduction and epithelial cell integrity. Four haplotypes have been identified, but only D haplotypes were associated with UC and CD in a European cohort[26]. Another variant of this gene (rs37462) was found in Japanese people with CD[27]. The haplotype characterized by the haplotype-tagging single nucleotide polymorphisms (SNP) G113A called haplotype D, was found substantially over-transmitted in patients with IBD.

DRUG TRANSPORTER

The multidrug-resistance (MDR-1) gene encodes the drug efflux pump P-glycoprotein 170 (Pgp-170). Various polymorphisms have been identified within MDR-1: a

mutation C3435T in exon 26 and a mutation G2677T in exon 21 have been correlated with altered Pgp expression and function in humans. Overexpression of MDR-1 leads to an increased efflux of drugs and decreased cytoplasmic drug concentrations. Several drugs, including glucocorticoids, are known Pgp-170 substrates. Farrell *et al*[28] showed that MDR was significantly elevated in CD and UC patients who required bowel resection and proctocolectomy after failed medical therapy. Variant C3435T was related to the presence of pancolitis in patients with UC in Scotland[29]. However, the frequency of SNPs is low and is different among populations, with the exception of three SNPs in exon 12 (C1236T), exon 20 (G2677T/A) and exon 26 (C3435T), and some of them are correlated with different diseases and clinical characteristics[30].

CELL ADHESION

Cell surface adhesion molecules conveying leukocyte-endothelial interactions, govern homing of activated inflammatory cells into gut. Extravasation and migration into the site of inflammation are mediated by integrins and selectins, and these molecules are increased in IBD patients. There are targeting therapies against adhesion molecules in clinical trials to date including natalizumab (integrin α4 subunit) and MLN-02 (selective adhesion molecule blocker for integrin $\alpha 4\beta 7$). In Japanese patients with IBD, the intercellular adhesion molecule-1 (ICAM-1) K469 allele is associated with CD and UC[31].

CONCLUSION

There are increasing numbers of genetic markers associated with the development of IBD at different levels: innate immunity, antigen presentation, epithelial integrity, drug transporter and cell adhesion that contribute, in genetic susceptibility, to the development of IBD in conjunction with environmental and immunological factors.

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TOPIC HIGHLIGHT

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Pouchitis

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Abstract

While restorative proctocolectomy with ileal pouch-anal anastomosis has significantly improved the quality of life in patients with underlying ulcerative colitis who require surgery, complications can occur. Pouchitis as the most common long-term complication represents a spectrum of disease processes ranging from acute, antibiotic-responsive type to chronic antibiotic-refractory entity. Accurate diagnosis using a combined assessment of symptoms, endoscopy and histology and the stratification of clinical phenotypes is important for treatment and prognosis the disease. The majority of patients respond favorably to antibiotic therapy. However, management of chronic antibiotic-refractory pouchitis remains a challenge.

Key words: Classification; Complication; Ileal pouch; Inflammatory bowel disease; Restorative proctocolectomy

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INTRODUCTION

Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) has become a part of standard surgical treatment for patients with ulcerative colitis (UC) or familial adenomatous polyposis (FAP). Despite advances in medical therapy, approximately 30% of patients with UC eventually require total proctocolectomy[1]. Restorative proctocolectomy with IPAA has the following advantages: (1) gastrointestinal continuity is reestablished with IPAA, (2) the procedure helps improve symptoms of patients and health-related quality of life, (3) the majority of patients with IPAA can avoid UC-related medications, particularly immunomodulators and biological agents and their associated potential adverse effects, and (4) IPAA with proctocolectomy substantially reduces the risk for dysplasia or cancer. However, adverse outcomes or complications often occur after surgery. Common long-term inflammatory and functional complications of restorative proctocolectomy are pouchitis, Crohn's disease (CD) of the pouch, cuffitis (inflammation in the rectal muscular cuff), and irritable pouch syndrome (IPS). Pouchitis likely represents a spectrum of disease processes ranging from acute antibiotic-responsive entity to chronic antibiotic-refractory type. Accurate diagnosis and classification of pouchitis are important for its proper management and prognosis.

INCIDENCE AND PREVALENCE

Pouchitis, a nonspecific inflammatory condition at the ileal pouch reservoir, is the most common long-term complication in patients with IPAA which significantly affects patients' quality of life[2]. Reported cumulative frequency rates of pouchitis 10-11 years after IPAA surgery range from 23% to 46%[3,4]. It is estimated that approximately 50% of patients who have undergone IPAA surgery for UC will develop at least one episode of pouchitis[5]. The estimated incidence within 12 mo after ileostomy was as high as 40% in a European study[6]. The discrepancy in the reported cumulative frequencies from different institutions likely results from diagnostic criteria used (e.g., diagnosis made based on symptom assessment alone or on a combined assessment of symptoms, endoscopy, with or without histology), intensity of follow-up with pouch endoscopy, and inclusion or exclusion of other inflammatory or functional disorders of the pouch and surgery related conditions (such as abscess, fistula, and sinus of the pouch).

ETIOLOGY AND PATHOGENESIS

Pouchitis almost exclusively occurs in patients with underlying UC and is rarely seen in patients with FAP[7,8]. Although the etiology and pathogenesis of pouchitis are

not entirely clear, bulk of evidence points towards an abnormal mucosal immune response (innate and adaptive) to altered microflora in the pouch leading to acute and/or chronic inflammation[6,9,10,11,12,13]. The prevailing theory holds that pouchitis results from an overgrowth of certain commensal bacteria[9,13,14,15]. Pouchitis only develops after ileostomy, i.e., the pouch mucosa starts to expose fecal stream. Manipulation of microflora with antibiotic or probiotic therapy resulting in improvement in patients with pouchitis provides additional evidence of involvement of microflora in the pathogenesis of pouchitis.

Immune mechanisms for pouchitis have been extensively studied in a similar fashion to that for inflammatory bowel disease. There are overlaps in tissue cytokine profiles between pouchitis and UC. However, pouchitis is not simply a duplication of the disease process seen in UC. The role of T-cell-mediated intestinal immunity in the pathogenesis of pouchitis is not entirely clear and is likely secondary to alterations in pouch microflora. Alterations in the macrophage and T cell subpopulations have been postulated in the process of pouchitis[16,17,18]. Increased T-cell activation and proliferation have been demonstrated in pouchitis, as evidenced by an increased expression in activation markers, such as CD25, CD30, and CD27[18]. As a result of activation of T cells and other immune cells, production of cytokines is up-regulated. Abnormal cytokine profiles have been reported in pouchitis including a deregulated production of proinflammatory and immunoregulatory cytokines[19]. Proinflammatory cytokines, such as TNF-α, are released at a great extent in the inflamed mucosa by macrophages and monocytes, leading to tissue injury, and are considered to be involved in pouchitis as a secondary pathophysiologic mechanism[19]. As in UC, the production of other inflammatory mediators including cytokines (such as IL-1β, IL-6, and IL-8)[20,21,22,30], cell adhesion molecules (such as E selectin and intercellular adhesion molecule-1)[23], platelet-activating factor[24], lipoxygenase products of arachidonic acids (such as leukotriene B4 and prostaglandin E2)[25], proinflammatory neuropeptides[26], macrophage inflammatory protein (MIP) 2α, matrix metalloproteinase (MMP)-1[21,27], MMP-2[21,27,28], MMP-9[28], MRP-14[21], and inducible nitric oxide[28], is also increased. Abnormalities in immunoregulatory cytokines such as IL-2, and interferon-γ[18,29], IL-4[29], and IL-10 are also seen in pouchitis. Imbalance between proinflammatory and immunoregulatory cytokines has been described in patients with pouchitis[30]. Abnormalities of T cells and other immune cells may not explain the whole mechanism of pouchitis. It is likely that such abnormalities are nonspecific and secondary in nature. Inconsistent results in the studies of immune cells and inflammatory mediators in pouchitis reflect the complexity in pathogenesis of the disease.

There are few published studies addressing the interplay between microflora and mucosal immune system in pouchitis. Exposure of peripheral blood and lamina propria lymphocytes *ex vivo* to sonicated flora from pouchitis induces more intense proliferation as compared with sonicates from healthy pouches. *In vitro* pretreatment of the sonicate preparation of pouch flora with metronidazole abolishes the stimulating ability[31]. Bacterial sonicates from a heterologous but healthy pouch do not stimulate lymphocyte proliferation[31]. The greater stimulatory effect of sonicates from pouchitis suggests that certain microflora may predominantly present in inflamed pouch mucosa and these microflora may be potentially pathogenic in activation of local mononuclear cells[31].

One of the most intriguing aspects of pouchitis is that it occurs almost exclusively in patients with underlying UC. Interestingly, there are similarities in terms of clinical presentations and immunological abnormalities between pouchitis and UC, suggesting that a subset of pouchitis may actually represent the recurrence of a UC-like disease in the ileal pouch. The theory of recurrent UC is supported by several lines of evidence. With the presence of stasis in the pouch, exposure to fecal contents and an increased microbial load could cause inflammatory changes leading to morphological alterations in the ileal pouch mucosa mimicking colon epithelia in UC[24]. Colonic metaplasia of the pouch mucosa seems to be a nonspecific adaptive response to the new luminal environment[24]. Colonic metaplasia characterized by villous blunting, crypt cell hyperplasia, and colon epithelium-specific antigens such as human tropomyosin 5, may increase the risk for pouchitis[32]. A similar alteration in mucin glycoproteins occurs in pouchitis as seen in UC[33]. It is possible that the altered glycoproteins are more susceptible to enzymatic degradation by bacteria, making the mucus barrier less resistant[34]. Additionally, some patients with pouchitis have the same extra-intestinal manifestations (such as arthralgia and primary sclerosing cholangitis or PSC) as those seen in patients with UC[35]. Smoking tends to have a protective effect against the development of pouchitis as it does against UC[36].

RISK FACTORS

Risk factors and potential predictors for pouchitis have been extensively studied. The implications of these studies include identification of etiopathogenetic factors, provision of strategies for modification of certain risk factors, and prediction of pouch outcome. Genetic polymorphisms such as those of IL-1 receptor antagonist[38,39,40] and NOD2/CARD15[40] may increase the risk for pouchitis. The reported risk factors for pouchitis also include non-carrier status of TNF allele 2[39], extensive UC[4,41,42], backwash ileitis[41], pre-proctocolectomy thrombocytosis[43], extra-intestinal manifestations, especially PSC[3,35,44,45], the presence of serum perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA)[46,47], being a non-smoker[36,42,48], and use of non-steroidal anti-inflammatory drugs (NSAID)[42,48]. In addition to p-ANCA, the presence of serologic markers, anti-*Saccharomyces cervesiae* antibodies to CD-related antigen from *Pseudomonas fluorescens* or outer membrane porin C of *Escherichia coli* in patients with pre-operative indeterminate colitis appears to be associated with persistent inflammation of the pouch after restorative proctocolectomy[49]. Acute and chronic pouchitis may have different risk factors[42].

It appears that few studies came up with the same risk factors. This inherent discrepancy among the studies may be contributed to the following factors: (1) small *vs* large

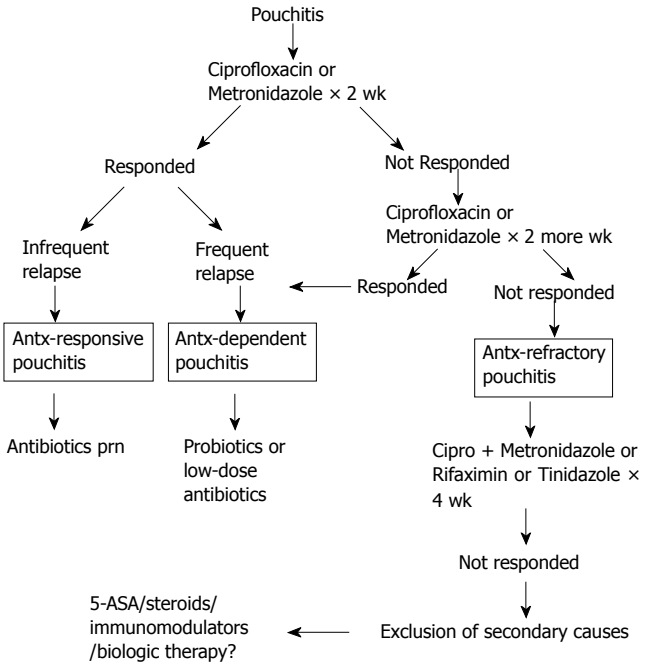

Figure 1 Classification and treatment algorithm.

sample sizes were analyzed, (2) the number of variables and outcomes was studied, (3) univariable analyses *vs* multivariable analyses were used, (4) diagnostic criteria for pouchitis were used, (5) pouchitis was stratified into acute *vs* chronic entities, and (6) type of controls was compared.

CLINICAL PRESENTATIONS

Patients with pouchitis have a wide range of clinical presentations, including increased stool frequency, urgency, tenesmus, incontinence, nocturnal seepage, abdominal cramping, and pelvic discomfort. While bloody bowel movements are uncommon in typical pouchitis, patients with IPAA with or without pouchitis can have iron deficiency anemia[50,51]. Patients with severe pouchitis occasionally present with fever, dehydration, malnutrition which may require hospitalization. Patients may have predominant extra-intestinal symptoms such as arthralgia and uveitis. These symptoms, however, are not specific and can present in disorders of the pouch other than pouchitis, such as cuffitis, CD of the pouch, proximal small bowel bacterial overgrowth, and IPS.

DIAGNOSTIC EVALUATIONS

Making diagnosis of pouchitis should not solely rely on presenting symptoms. The severity of symptoms does not necessarily correlate with the degree of endoscopic or histologic inflammation of the pouch[52,53]. A combined assessment of symptoms, endoscopic and histologic features is the key to making an accurate diagnosis and it is necessary to differentiate pouchitis from other inflammatory and non-inflammatory disorders of the pouch such as cuffitis, pouch stricture, pouch sinus, and IPS. There are no universally accepted diagnostic criteria for pouchitis. For clinical trials, the 18-point pouchitis disease activity index (PDAI) is most commonly used in the diagnosis of pouchitis and measurement of disease activity[54].

Pouch endoscopy yields valuable information on severity and extent of mucosal inflammation, presence or absence of concurrent ileitis or cuffitis, and structural abnormalities such as strictures, sinuses, and fistula openings. In addition, pouch endoscopy with segmental biopsy is the tool for dysplasia surveillance and can deliver effective therapy, including stricture dilation. Histopathology is invaluable for the detection of dysplasia, viral inclusion bodies of cytomegalovirus infection, granulomas, pyloric gland metaplasia, mucosal prolapse, and ischemic changes. It should be pointed out that villous blunting and an increased number of mononuclear cells in the lamina propria can be a part of "normal" adaptive changes of pouch mucosa to fecal stasis in the pouch which does not necessarily indicate pouchitis.

In cases of suspected complicated pouchitis, imaging studies such as contrasted pouchography, CT and MRI are typically used to assess the presence of mucosal and transmural disease activity within and around the pouch[55]. Wireless capsule endoscopy appears safe in patients with IPAA, which has been used for diagnostic evaluation in patients with chronic pouchitis[56] or anemia[57]. For patients with symptoms of dyschezia and feeling of incomplete evacuation, anal pouch manometry may be used to identify functional abnormalities such as paradoxical contractions.

CLINICAL CLASSIFICATION

Pouchitis likely represents a disease spectrum from acute, antibiotic-responsive type to chronic, antibiotic-refractory entity. From various perspectives, pouchitis can be categorized into: (1) idiopathic vs secondary based on etiology, (2) remission *vs* active based on disease status, (3) acute *vs* chronic based on disease duration, (4) infrequent episodes *vs* relapsing or continuous based on disease course, and (5) responsive *vs* refractory based on response to antibiotic therapy[58]. A subpopulation of patients has pouchitis associated with identifiable and modifiable causes (namely secondary pouchitis), such as *Clostridium difficile*[59,60] or cytomegalovirus[61,62] infection, and regular use of NSAID[63].

While the majority of patients with pouchitis respond favorably to antibiotic therapy particularly at initial stages of the disease, some patients develop pouchitis refractory to regular antibiotic treatment. This leads to another useful clinical classification based on the response to antibiotic therapy[64]. Analogous to the classification of UC according to the response to or dependency on corticosteroids, pouchitis can be classified as antibiotic-responsive, antibiotic-dependent, and antibiotic-refractory pouchitis[48,64] based on the manner of the patients' response to antibiotics (Figure 1).

TREATMENT

As the majority of patients develop acute pouchitis within the first year after IPAA[65], VSL#3® containing 4 strains of *Lactobacillus*, 3 *Bifidobacterium* species, and *Streptococcus*

salivarius subsp. *Thermophillus* was evaluated for the primary prophylaxis for the initial episode of pouchitis. Two of 20 patients (10%) treated with VSL#3® developed pouchitis within 12 mo after IPAA, while 8 of 20 patients (40%) experienced pouchitis in the placebo group during the same period of time[6].

The management and prognosis vary in different types of pouchitis (Figure 1). For antibiotic-responsive pouchitis, the first-line therapy includes a 14-d course of oral metronidazole (15-20 mg/kg per day) or ciprofloxacin (1000 mg/d)[66,67]. A randomized trial of ciprofloxacin and metronidazole showed that patients treated with ciprofloxacin experience significantly greater reductions in the PDAI scores and fewer adverse effects than those treated with metronidazole[67]. Other agents have been reported in open-labeled trials including tetracycline, clarithromycin, amoxicillin/clavulanic acid, doxycycline, rifaximin, and budesonide enemas[68], alicaforsen enemas, an anti-sense inhibitor of intercellular adhesion molecule-1[69], and AST-120, a highly adsorptive, porous, carbon microspheres[70].

Patients with antibiotic-dependent pouchitis often require long-term maintenance therapy with either antibiotics or probiotics to keep disease in remission. A randomized trial of VSL#3® at a dose of 6 g/d was conducted for the secondary prophylaxis for relapse of pouchitis, after remission was induced by oral ciprofloxacin (1000 mg/d) and rifaximin (2000 mg/d). During the 9-mo trial in 40 patients with relapsing pouchitis, only 15% in the probiotic group relapsed while 100% in the placebo group relapsed[11]. A separate randomized trial of VSL#3® in patients with antibiotic-dependent pouchitis showed that 17 of 20 patients (85%) in the VSL#3® group maintained clinical remission, compared to remission in 1 of 16 patients (6%) in the placebo group[12]. However, in a recent post-market open-labeled trial of VSL#3® in 31 patients with antibiotic-dependent pouchitis, patients received 2 wk of treatment with ciprofloxacin followed by VSL#3®[71]. After 8 mo, 6 of the 31 patients (19%) were still taking VSL#3® and the remaining 25 patients (81%) stopped the agent mainly because of lack of efficacy or development of adverse effects[71].

Antibiotic-refractory pouchitis which is often difficult to treat, is a common cause of pouch failure. Since the patients typically do not respond to full-dose, single-agent antibiotic therapy, it is important to investigate contributing causes (in secondary pouchitis) related to failure to antibiotic therapy. Secondary causes of refractory disease include use of NSAID, concurrent *Clostridium difficile* or cytomegalovirus infection, celiac disease and other autoimmune disorders, cuffitis, CD of the pouch, pouch ischemia, and inflammatory polyps of the pouch[72]. There are no randomized trials in the literature for this category of pouchitis. For patients without obvious causes, treatment options include a prolonged course of combined antibiotic therapy, 5-aminosalicylates, corticosteroids, immunosuppressive agents or even biological therapy. Regimens reported in open-labeled trials include combined ciprofloxacin (1000 mg/d) with rifaximin (2000 mg/d)[73] or metronidazole (1000 mg/d)[74] or tinidazole (1000-1500 mg/d) for 4 wk[75]. However, maintenance of remission in this group of patients after the induction therapy with dual antibiotics remains a challenge[76]. Anti-inflammatory agents, immunomodulators, and biological therapy have been used to treat pouchitis. These agents include bismuth carbomer enemas, short-chain fatty acid enemas, and glutamine enemas, mesalamine enemas, oral budesonide[77], 6-mercaptopurine, and infliximab.

NATURAL HISTORY AND PROGNOSIS

The natural history of pouchitis is not entirely clear. In a study consisting of 100 consecutive UC patients who had restorative proctocolectomy with IPAA, 32 patients developed pouchitis, 5 had chronic refractory pouchitis, 2 of them had pouch failure after pouch resection[58]. Few studies were performed to identify the natural history of pouch and pouchitis. Patients with initial pouchitis almost uniformly respond to antibiotic therapy. However, relapse of pouchitis is common. Of the patients with acute pouchitis, 39% have a single acute episode that responds to antibiotic therapy whereas the remaining 61% of patients develop at least one recurrence[35]. Approximately 5% to 19% patients with acute pouchitis develop refractory or rapidly relapsing symptoms[78-80]. Here is a common scenario: the more frequent the episodes of pouchitis a patient has, the more often the antibiotic therapy is administered, the less likely the patient maintains favorable response to the treatment. The course of antibiotic-responsive pouchitis could evolve into antibiotic-dependent pouchitis followed by antibiotic-refractory pouchitis. Chronic refractory pouchitis is one of the most common causes for pouch failure. Although PSC is a risk factor for pouchitis[3,44,45], liver transplantation with post-transplant use of immunosuppressive agents does not appear to have adverse effects on the course of pouchitis[81,82]. In addition, chronic inflammation of the pouch and cuff may pose an increased risk of developing dysplasia or cancer[83,84].

In summary, pouchitis is the most common long-term adverse sequela of IPAA after restorative proctocolectomy. The natural history of pouchitis is yet to be defined. Patients with pouchitis can have a wide range of clinical presentations, disease courses, and prognoses. Accurate diagnosis and classification of pouchitis are the keys to appropriate management. Treatment of pouchitis is largely antibiotic-based. Maintenance of remission in antibiotic-dependent pouchitis and management of antibiotic-refractory pouchitis are a challenge. Secondary causes for refractory pouchitis should be excluded.

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BASIC RESEARCH

Effect of *Scutellariae Radix* extract on experimental dextran-sulfate sodium-induced colitis in rats

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Abstract

AIM: To investigate the effect of *Scutellariae Radix* extract (SRE) on ulcerative colitis (UC) in rats induced by dextran-sulfate sodium (DSS).

METHODS: Colitis was induced in male Sprague-Dawley (SD) rats (170-180 g) by 4% dextran sulfate sodium (DSS, wt/v; MW 54000) in drinking water for 8 d. The treated rats received 4% DSS and SRE orally (100 mg/kg per day). Control rats received either tap water or SRE only. Macroscopic assessment which included body weight changes, fecal occult blood and stool consistency were determined daily. At the appointed time, the rats were sacrificed and the entire colons were removed. The colon length and the myeloperoxidase (MPO) activity were measured. The severity of colitis was graded by morphological and histological assessments. The ion transport activity of the colonic mucosa was assessed by electrophysiological technique.

RESULTS: Rats treated with oral administration of 4% DSS regularly developed clinical and macroscopic signs of colitis. Treatment with SRE relieved the symptoms, including the reduction in body weight, shortening and ulceration of the colon. Administration of SRE also significantly reduced the histological damage induced by DSS. Moreover, the *Isc* responses of the colonic mucosa to forskolin were suppressed after the induction of colitis. The stimulated ion transport activity of DSS-rats treated with SRE displayed significant improvement in the secretory responsiveness.

CONCLUSION: SRE was effective in treating acute DSS-induced ulcerative colitis, as gauged by reduced clinical disease, improved macroscopic and histological damage scores, and enhanced recovery of normal colonic secretory function.

Key words: Ulcerative colitis; *Scutellariae Radix*; Inflammatory bowel disease; Colonic ion transport; Traditional Chinese medicine

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INTRODUCTION

Ulcerative colitis is a worldwide, chronic, idiopathic, inflammatory bowel disease (IBD) of the rectal and colonic mucosa. In the past, this disease was thought to occur infrequently in the Asia Pacific region. However, new evidence is showing that IBD is on the rise in the region, including in Hong Kong and mainland China[1-3]. Although glucocorticoid and salicylazosulfapyridine have been mainly used for the treatment of this disease, their side effects remain a major clinical problem. Therefore, there is an increasing interest in using traditional Chinese medicines (TCM) as alternative therapy in addition to the conventional therapies that are used to treat UC[4].

The dried root of *Scutellaria baicalensis* Georgi (*Scutellaria Radix*, common name Huangqin) is widely used in TCM. It is officially listed in the Chinese Pharmacopoeia and is one of the most widely used Chinese herbal medicines for the treatment of bacterial infection of the respiratory and gastrointestinal tract. In Japan and China, *Scutellariae Radix* has been employed for centuries as an important medicine to treat chronic inflammatory and ulcerative disease. The main components of *Scutellariae Radix* (and of all *Scutellaria* species) are baicalein, baicalin and wogonin.

Scutellariae Radix and its major flavonoids possess multiple biological and pharmacological effects, including anti-inflammation[5], anti-viral[6], anti-tumor[7], anti-proliferative[8] and anti-bacterial[9], *etc*. Recent studies also suggest that *Scutellariae Radix*-containing TCM formula, such as Orengedoku-to (Huang Lian Jie Du Tang) may have therapeutic potential against murine colitis[10-12].

In this study, an experimental model of UC was established in SD rats using DSS. The effect of the total extract of *Scutellariae Radix* on DSS-rats was evaluated using macroscopic, histological, biochemical and electrophysiological assessments.

MATERIALS AND METHODS

Materials

Male SD rats, initially weighing 170-180 g, were housed five per cage and maintained in an animal holding room controlled at a constant temperature of 24℃ ± 2℃ with a relative humidity of 70% ± 5% and a 12 h light-dark cycle. Animals were fed on a standard pellet chow with free access to fresh tap water. The study was approved by the Animal Research Ethics Committee of our university. DSS was obtained from MP Biochemicals Inc. Hexadecyltrimethylammonium bromide, forskolin were obtained from Sigma. SRE was purified from the ground roots of *S. baicalensis* Georgi with hexane, acetone, and finally, with methanol as described previously[13,14].

Experimental design

The induction of colitis was modified from a previously described work[15]. The experiment lasted for 8 d. The rats were randomly divided into four groups. In the DSS Group, 4% DSS in drinking water *ad libitum* was given from d 0 to d 7. In DSS + SRE group, SRE (100 mg/kg per day) was orally administrated with exposure to 4% DSS drinking water. In the normal control group (Ctr), the rats had free access to a water bottle containing tap water. In Ctr + SRE group, SRE alone (100 mg/kg per day) was administered orally to the rats. In this model, the colonic damage was evaluated using macroscopic, histological, electrophysiological and biochemical assessments (see below). From d 3 to d 7, rats were sacrificed by CO_2 asphyxiation on each day. Postmortem, the entire colon was removed from the cecum to the anus and placed on an ice cold plate and cleaned of fat and mesentery. The length of each specimen was measured, which is an indirect marker of inflammation. The colon was divided into three parts (proximal, middle and distal) based on total length: 10% regions from three parts were fixed for histological examination; the distal portion was used for electrophysiological studies; and the adjacent distal 10% was snap-frozen in liquid nitrogen for later quantification of MPO activity.

Macroscopic assessment

Animals were checked daily for the three main clinical symptoms-body weight changes, stool consistency and fecal occult blood. Weight loss is usually observed in animals with colitis, thus body weight changes recorded could be an indicator for the severity of colitis. The colonic damage was quantified by a clinical scoring system assessing stool consistency and rectal bleeding[16]. For stool consistency, 0 points were given for well formed pellets, 2 points for pasty and semiformed stools that did not stick to the anus, and 4 points for liquid stools that did stick to the anus. Bleeding was scored 0 for no blood in hemoccult, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were added, forming a total clinical score that ranged from 0.0 (healthy) to 8.0 (maximal activity of colitis).

Histological assessment

For light microscopy, we used tissue samples from three parts (distal, middle and proximal) of colon of each animal fixed in 4% (40 g/L) buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 μm on a rotary microtome, mounted on clean glass slides and dried overnight at 37℃. The sections were cleaned, hydrated, and stained with hematoxylin and eosin (HE) for histological evaluation of inflammatory infiltrate and tissue damage, according to standard protocols, and the slides were coded to prevent observer bias during evaluation. All tissue sections were examined in a blinded fashion by two investigators. Histological damage was scored using the criteria of Siegmund B *et al*[17] which considers the inflammatory infiltrate (maximum score = 3) and tissue damage (maximum score = 3). Photographs of colon samples were digitized using a ZEISS Axioskop 2 plus camera. Analysis of the figures was carried out with Axio Vision 3.1 image analysis program.

Electrophysiological assessment

Mucosal ion transport was examined in colonic segments (approximately distal colon) mounted in Ussing chambers according to a well established protocol in our laboratory[18]. In brief, tissues (surface area = 0.45 cm^2) were bathed in 20 mL of warm (37℃), oxygenated Krebs buffer. The spontaneous potential differences were maintained at 0 mV by a voltage clamp amplifier (Physiologic Instruments), and the short-circuit current (I_{SC} in μAcm^{-2}) was continuously measured as an index of electrogenic ion transport. A transepithelial potential difference of 1 mV was applied periodically, and the resultant change in current was used to calculate the transepithelial resistance (R_t) using Ohm's law. Stimulated ion transport was evoked by the addition of an adenylate cyclase-activating agent, forskolin (1 μmol/L), to the mucosal bathing solution. In all instances, the effect of the treatment was recorded as the maximum change in I_{SC} (ΔI_{SC}) to occur within 5 min.

Biochemical assessment

The MPO activity was determined following a published protocol[19]. Briefly, frozen tissue samples were weighed and suspended in potassium phosphate buffer (20 mmol/L, pH 7.4) at a ratio of 50 mg tissue to 1 mL of buffer. Tissue was homogenized by a polytron tissue homogenizer three times for 20 s, and homogenate was decanted into sterile

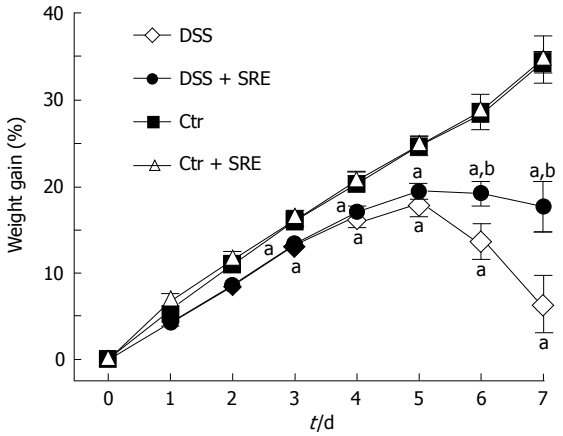

Figure 1 Figure showing the weight gain percentage (%) in different groups of rat from d 0 to d 7. Body weight was assessed daily and expressed as percentage increase of basal body weight. Values are expressed as the means ± SE, (n = 8), $^{a}P < 0.05$ *vs* Ctr group, $^{b}P < 0.05$ *vs* DSS group, one-way ANOVA followed by Tukey multiple comparison test.

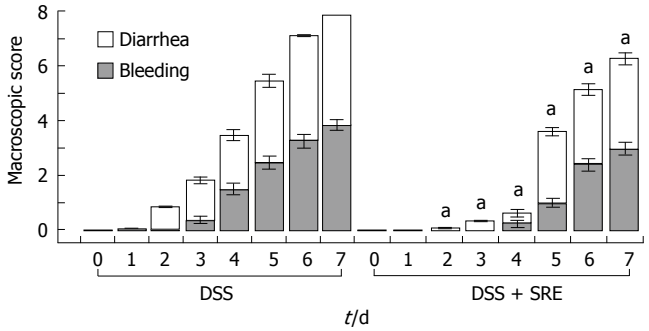

Figure 2 Ameliorative effect of SRE treatment on the time-course changes in the macroscopic score over the 8-d experimental period. The macroscopic score of Ctr and Ctr + SRE groups is 0 (data not shown). $^{a}P < 0.05$ *vs* DSS group. Non-parametric data are expressed as the means ± SE, Kruskal-Wallis One Way Analysis using Student-Newman-Keuls method (n = 6-8).

Eppendorf tubes and centrifuged at 10000 × g for 10 min at 4℃. The pellet was then resuspended in 1 mL potassium phosphate buffer (50 mmol/L, pH 6.0) containing 5 mg/mL hexadecyltrimethylammonium bromide (TMB) at a tissue concentration of 50 mg/mL. Samples were sonicated three times for 10 s, freeze-thawed three times, and centrifuged at 10000 × g for 5 min at 4℃. The reaction was started by mixing 20 μL of the supernatant at 25℃ with 30 μL TMB. After 110 s, the reaction was terminated by addition of 50 μL of 0.18 mol/L H_2SO_4. The change in absorbance was read at 450 nm. MPO activity (1 unit) was expressed as the amount of enzyme necessary for the degradation of 1 μmol H_2O_2/min per 100 mg tissue at 25℃.

Statistical analysis

All data are presented as means ± SE. One way ANOVA followed by post hoc statistics with Tukey test was used for statistical evaluation of the parametric data. Non-parametric data was analyzed by Kruskal-Wallis One Way Analysis using Student-Newman-Keuls method. $P < 0.05$ was considered as statistically significant. The values of n refer to the number of experiments undertaken using different rats. Non-parametric data are expressed as the means ± SE.

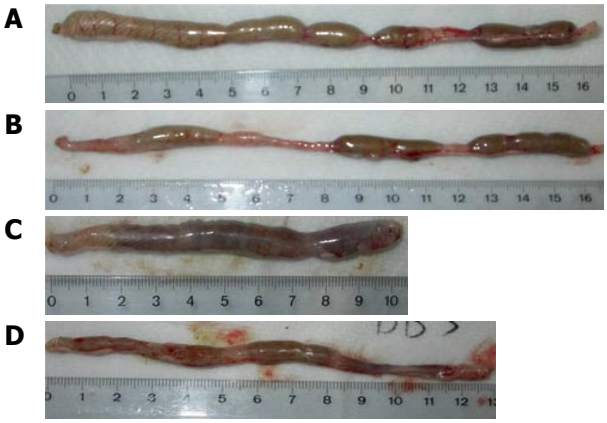

Figure 3 Macroscopic view of the colon showing the changes in colon length in different groups of rat on d 7. Not much difference was observed between the colon of the Ctr (**A**) and Ctr + SRE group (**B**), but significant difference could be found in the DSS group (**C**) which displayed extensive hyperemia and edema. In the DSS + SRE group, the shortening of colon was less severe (**D**) when compared with the control (**A**).

RESULTS

Macroscopic assessment

The weight gain % over the entire study period is shown in Figure 1. The weight gain % of rats in DSS group and DSS + SRE group were significantly lower than the Ctr group and Ctr + SRE group from d 3 until the end of experiment. The body weight of rats in DSS group then dramatically decreased from d 5 onwards. However, in the case of DSS + SRE group, the weight gain % became stabilized and the weight loss was found to be less severe than DSS group from d 6 to d 7. Figure 2 shows the macroscopic score recorded throughout the experimental period in DSS and DSS + SRE groups. Oral administration of SRE resulted in a significant reduction in the clinical activity of colitis compared with DSS-treated rats. Moreover, the occurrences of those clinical signs were found to be delayed in DSS + SRE group.

The colon length is a useful indication of colitis and is, therefore, measured as a marker of inflammation (Figure 3). After 8 d treatment with DSS in drinking water, there was a significant shortening of the colon length (Figure 3C) compared with the Ctr group (Figure 3A) and the Ctr + SRE group (Figure 3B). The oral administration of SRE significantly improved this inflammatory marker (Figure 3D). On d 7, the colon length of DSS group (11.2 cm ± 0.4 cm, n = 10) was significantly shorter than the control group (16.8 cm ± 0.6 cm, n = 8). The colon length of DSS-rats treated with SRE (13.5 cm ± 0.4 cm, n = 9), however, was significantly longer than that of untreated rats.

Histological assessment

Histological damage was evaluated by the grading method described above in Materials and Methods. The occurrence of UC was confirmed on the basis of histological damage and inflammatory infiltrate as shown in Figure 4. Figure 5 summarized the damage scores from DSS rats and DSS rats treated with SRE. The microscopic score of samples

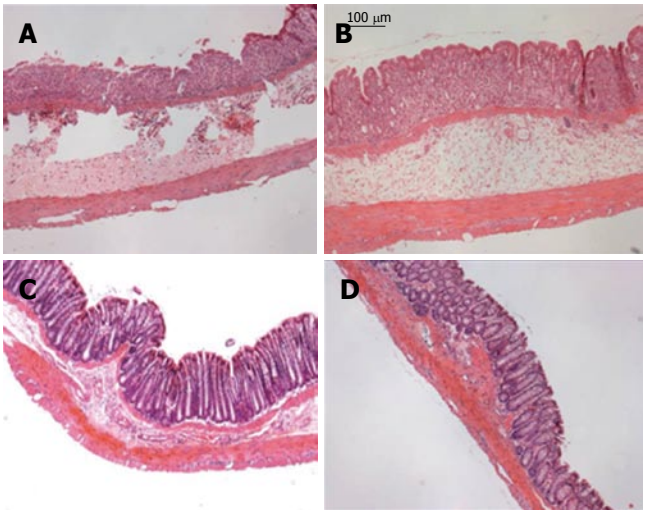

Figure 4 Histological sections from different groups of rats on d 7. **A**: DSS group showing extensive ulceration with a severe inflammatory cell infiltrate; **B**: DSS + SRE group showing recovery in the inflammatory cell infiltration with less severe ulceration; **C**: Non-colitic Ctr group showing the normal histology of the colon; **D**: Ctr + SRE group showing the normal morphology of the colon. (HE staining; original magnifications, x 100).

from different regions (distal, middle and proximal) of colon were not significantly different from each other (data not shown). The histological index began to increase on d 3 in both groups. However, the rats with SRE administration showed a significantly lower value than the untreated animals on d 6 and d 7.

Electrophysiological assessment

Electrogenic ion transport function was assessed by stimulating the colon with an adenylate cyclase activator, forskolin (1 μmol/L), using short-circuit current measurement technique (Figure 6). Under the experimental conditions, the increase in I_{SC} is mainly due to the cAMP-mediated Cl^- secretion *via* the cystic fibrosis transmembrane conductance regulator (CFTR)[18]. The basal I_{SC} and R_t in the tissues were recorded and these parameters were not significantly different (data not shown) when tissues from different groups were compared. On d 7, the secretory response to the cAMP-elevating agent was significantly diminished in tissues from rats treated with DSS (ΔI_{SC} = 4.94 ± 1.65 μAcm^{-2}, n = 6) when compared with Ctr (ΔI_{SC} = 60.97 ± 7.62 μAcm^{-2}, n = 8) and Ctr + SRE (ΔI_{SC} = 61.48 ± 6.78 μAcm^{-2}, n = 7). On the other hand, the reduced responsiveness was ameliorated in the colitic rats with SRE administration (ΔI_{SC} = 20.67 ± 3.30 μAcm^{-2}, n = 9).

Biochemical assessment

The MPO activity in the groups without DSS treatment remained at a low value throughout the entire experiment and there was no effect of SRE on the control (d 7: Ctr 0.09 ± 0.01 mU/mg, n = 6; Ctr + SRE 0.06 ± 0.0003 mU/mg, n = 5). In comparison, rats with colitis were accompanied by a significant increase in MPO activity (d 7: 0.52 ± 0.07 mU/mg, n = 8). On d 7, the increase in MPO activity (0.34 ± 0.05 mU/mg, n = 8) was significantly reduced in rats treated with SRE.

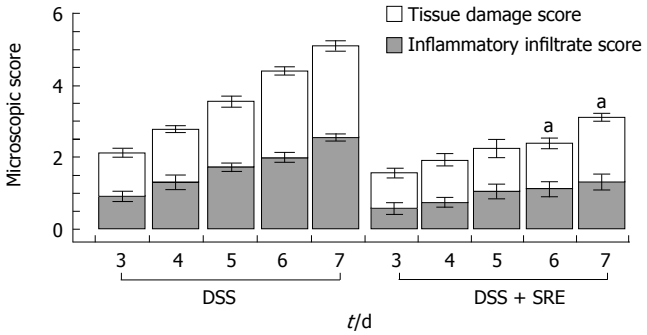

Figure 5 Ameliorative effect of SRE treatment on the time-course changes in the microscopic score from d 3 to d 7. The microscopic score of Ctr and Ctr + SRE groups is 0 (data not shown). $^aP < 0.05$ *vs* DSS group. Non-parametric data are expressed as the means ± SE, Kruskal-Wallis One Way Analysis using Student-Newman-Keuls method (n = 4-5).

DISCUSSION

Ulcerative colitis is an inflammatory disease that causes ulcerations of the mucosa in the colon. The incidence of UC is around 1 in 1000 people with a higher prevalence among Caucasians[20]. In the past, this disease has been considered to occur rarely in the Asia Pacific region, but recent evidence indicates that both UC and Crohn's disease (CD) are becoming increasingly prevalent in local populations[3]. For example, from 1991 to 2000, there has been a three-fold increase in the number of cases of UC in China[1]. In Hong Kong, from 1986-1989 to 1999-2001, there was also a three-fold increase in the incidence of CD in the Chinese population[2]. Although the etiology of IBD remains essentially unknown[21], results from many studies in human patients and animal models suggest that it may be related to an abnormal immune response in the gastrointestinal tract, possibly associated with genetic and environmental factors[22,23]. Although progress has been made in the overall management of UC, the pharmacological treatments that are available are still unsatisfactory. Glucocorticoids, sulfasalazine and immunosuppressive drugs have been mainly used for the treatment and maintenance of UC, but the side effects or toxicity of these drugs remain a major clinical problem[24]. As a result, there is an increasing interest in using TCM as alternative therapy in addition to the conventional therapies that are used to treat UC[25]. In Japan and China, the most commonly used alternative remedies are herbal and these have been widely used in patients with mild to moderate disease, as well as an adjunct to therapy in patients with moderate to severe disease[1]. Interestingly, a recent survey showed that one-third of both Chinese and Caucasian IBD patients had used complementary and alternative medicines and therapies[4]. Several TCM formulae have been shown to possess an anti-colitic effect in rats[26-28]. Recent studies suggest that *Scutellariae Radix* may have a pharmacological effect against murine colitis[10-12] and therefore in this study we aim to further evaluate the therapeutic effect of SRE on DSS-induced rat colitis.

In the present study, 4% DSS in drinking water was administrated for 8 d to induce acute colitis in rats. All DSS-treated rats showed numerous clinical symptoms such as body weight loss, diarrhea, bloody stools and shortening

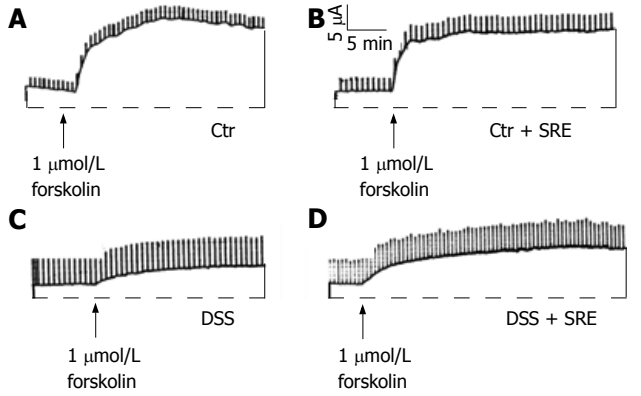

Figure 6 Representative tracings showing the change in I_{SC} evoked by forskolin in different groups of rat on d 7. All four groups showed an increase in ISC but with different magnitudes (**A-D**). The I_{SC} response was greatly reduced in the DSS group (**C**) when compared with the control (**A**). On the other hand, the reduction in secretory response appears to be ameliorated in the DSS + SRE group (**D**). The transient current pulses were the results of intermittently clamping the potential at 1 mV. The horizontal lines represent zero I_{SC}. The record is representative of at least six experiments.

of colon length. Body weight loss is a common symptom of UC because of the loss of body fluid and damage to the digestive system. In Ctr and Ctr + SRE groups, rats showed a steady increase in body weight throughout the experiment. In comparison, rats exposed to DSS had a lower rate of body weight increase followed by a dramatic decrease from d 5 onwards. Treatment with SRE to the colitic rats showed a less severe weight loss from d 6 to d 7. In DSS group, diarrhea and rectal bleeding occurred in d 1 and d 2, respectively. The SRE administration delayed the occurrence of both symptoms and with less severity. Colon shortening is always found in UC patients, which can act as an indirect marker of colonic inflammation. Although the colitic rats with SRE treatment also showed a decrease in colon length on d 7 when compared with the control, the shortening was much less severe than that of DSS rats. Taken together, the data suggested that SRE treatment could induce a decrease in the extent of colitis accompanied by reducing the severity and delaying the occurrence of the associated clinical symptoms.

Colitis induced by DSS was histologically characterized by severe disruption of tissue architecture, edema, a massive mixed immune cell infiltrate, ulceration and muscle thickening. To quantify the histological damage, we used a scoring system modified from a previous study[17]. We found that the overall histological damage of colitis was significantly reduced by the SRE treatment on d 6 and d 7 of the experiment. Together with the reduced MPO activity, our results showed that SRE treatment ameliorated the DSS-induced colitis possibly *via* its anti-inflammatory and protective effect against the colonic tissue damage.

The colonic epithelium, in addition to its absorptive and secretory properties, presents an efficient barrier to commensal flora and pathogens[29,30]. In fact, the secretion of water and electrolytes is one of the most important responses of the mucosa, purging the gut of offending agents and delivering anti-microbial mediators (e.g., antibodies) to the luminal surface[31]. In addition, mucus forms a gel layer covering the mucosal surface, and it has been hypothesized that changes in mucin structure and/or quantity may influence the protective functions of the mucosal surface, and affect the pathogenesis of IBD[32]. However, published data on the electrolyte transport mechanisms in an inflamed colon are inconsistent[33,34]. Some studies have documented the acute effects of immune and inflammatory agents in directly or indirectly stimulating intestinal anion secretion, while others do not support such a notion[35,36]. In most cases, however, disease models of colonic mucosa exhibit reduced ion transport responses to secretory agonists, especially in the setting of chronic inflammation[37-45].

The mechanism underlying the typical hyporesponsiveness of tissues from animal models of colitis has yet to be satisfactorily explained. For example, it has been reported that responsiveness to both Ca^{2+}- and cAMP-dependent secretagogues is reduced in mouse and rat colitis models when compared to normal tissue[37-45]. Similar reduction in secretory responsiveness, as measured by changes in I_{SC} has also been observed in tissue resections from patients with IBD[46,47]. It has been proposed that prolonged hyporesponsiveness to secretagogues is due to the upregulation of inducible nitric oxide synthase (iNOS), resulting in an ongoing synthesis of NO and chronic suppression of epithelial secretory function[41,42]. However, another recent study suggests that it may be related to a disturbance of the enteric nervous system resulting in defective mucosal cAMP production and inhibition of ionic secretion, although the epithelial secretory machinery (e.g., CFTR) appears to be normal[43]. Others suggested that prolonged hyporesponsiveness to secretagogues is due to the disruption of normal cholinergic control of ion secretion[40,48]. Nonetheless, intestinal secretion is an important component of mucosal defense. Reduced secretory responses will compromise the ability of the mucosal defense mechanism to clear bacteria, bacterial products, or antigens away from the epithelium, which may then predispose the colon to inflammation[49]. In this study, the I_{SC} responses of the colonic mucosa to forskolin were suppressed after the induction of colitis. Although the stimulated ion transport activity of DSS-rats treated with SRE was still reduced, they displayed improvement in the secretory responsiveness which may at least partly contribute to the therapeutic effect of SRE.

In summary, our findings indicated that SRE was effective in treating acute DSS-induced ulcerative colitis, as gauged by reduced clinical disease, improved macroscopic and histological damage scores, and enhanced recovery of normal colonic secretory function. The results support further evaluation of the therapeutic potential of SRE for the treatment of IBD.

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COMMENTS

Background

Scutellariae Radix, also known as Huangqin, is the dried root of *Scutellaria*

baicalensis Georgi (Lamiaceae). It is officially listed in the Chinese Pharmacopoeia and is one of the most widely used Chinese herbal medicines for the treatment of bacterial infection of the respiratory and gastrointestinal tract. In Japan and China, *Scutellariae Radix* has been employed for centuries as an important medicine. Although scientific evidence confirming the traditional use of *Scutellariae Radix* as an inflammatory modulator in experimental colitis is now accumulating, the physiological basis and the precise mechanism of action of *Scutellariae Radix* or its individual constituents remain largely unknown.

Research frontiers

Cytokine dysregulation is currently an important focus of both basic and clinical research in IBD, with recent immunologically based therapeutic interventions using highly specific agents demonstrating a promising clinical efficacy. The treatment of steroid-refractory Crohn's Disease with anti-TNF-α (infliximab) is an example of this kind of therapeutic approach. In addition, there is an increasing interest in using TCM as alternative therapy in addition to the conventional therapies that are used to treat IBD. However, there is still a paucity of scientific and clinical data so far to support the use of TCM in colitis patients. Therefore, there are unmet needs for further mechanistic, pharmacological and pharmacokinetic studies to delineate the biological basis underlying the therapeutic effects of TCM. More controlled clinical trials of the potential efficacy and safety of herbal TCM therapies are also required.

Innovations and breakthroughs

The authors showed for the first time that the therapeutic potential of *Scutellariae Radix* extract on experimental colitis may be related to the restoration of ion transport function of the colonic mucosa.

Applications

The results support further evaluation of the therapeutic potential of this herbal extract and its active component(s) for the treatment of IBD.

Terminology

Colonic ion transport: Intestinal fluid secretion is a passive process driven by osmotic forces generated by ion transport. In the colon, the main determinant of a luminally-directed osmotic gradient is the mucosal transport of chloride ions into the lumen. Intestinal secretion is an important component of mucosal defense. Reduced secretory responses will compromise the ability of the mucosal defense mechanism to clear bacteria, bacterial products, or antigens away from the epithelium, which may then predispose the colon to inflammation.

Peer review

This is an interesting paper which shows the anti-inflammatory effect of *Scutellariae Radix* on experimental colitis. It also demonstrates that TCM has a scientific and biological basis for its effectiveness.

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BASIC RESEARCH

Induction of apoptosis by artemisinin relieving the severity of inflammation in caerulein-induced acute pancreatitis

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Abstract

AIM: To observe the apoptosis and oncosis of pancreatic acinar cells and secondary inflammatory reaction in pancreatic tissue from rats with acute pancreatitis (AP), and the influences of artemisinin on them.

METHODS: AP was induced by 4 intraperitoneal injections of caerulein at 1 h intervals. To induce apoptosis, solution of artemisinin (50 mg/kg) was given intraperitoneally 1, 12, 24 and 36 h after the last caerulein injection. Histological examination of impairment of pancreatic tissue and detection of serum amylase were performed to evaluate the severity of acute pancreatitis. Apoptosis and oncosis were detected with acridine orange (AO) and ethylene dibromide (EB) staining. Caspase-3 and myeloperoxidase (MPO) activity were measured by colorimetric assay. Nuclear factor-kappa B (NF-κB) activation was detected by flow cytometry. Macrophage inflammatory protein-1α (MIP-1α) protein was measured by Western blot. Interleukin-1β (IL-1β) mRNA was detected by RT-PCR.

RESULTS: Addition of artemisinin increased the number of apoptotic cells (11.7% ± 1.4% *vs* 6.3% ± 0.7%, $P < 0.05$), while reduced the number of oncotic cells (13.0% ± 2.4% *vs* 17.5% ± 2.2%, $P < 0.05$). The activity of caspase-3 speeded up (1.52 ± 0.21 *vs* 1.03 ± 0.08, $P < 0.05$), the pancreas pathological impairment was relieved (3.0 ± 0.5 *vs* 4.0 ± 0.5, $P < 0.05$) and the level of serum amylase decreased (5642 ± 721 U/dL *vs* 7821 ± 653 U/dL, $P < 0.05$). The activation of NF-κB (29% ± 4.1% *vs* 42% ± 5.8%), MIP-1α protein (3.7 ± 0.5 *vs* 5.8 ± 0.7), MPO (0.52 ± 0.06 U/g *vs* 0.68 ± 0.09 U/g), IL-1β mRNA (1.7 ± 0.3 *vs* 2.4 ± 0.4) in the apoptosis inducing group was obviously decreased ($P < 0.05$).

CONCLUSION: Inducing apoptosis can relieve pathological impairment and inflammatory reaction in AP rats.

Key words: Pancreatitis; Apoptosis; Inflammation mediators; Chemokines; Artemisinin

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INTRODUCTION

Many factors lead to acute pancreatitis (AP). A series of cascade reactions of inflammatory mediators and over-activation of leukocytes are the important causes for systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS)[1]. Although anti-cytokine therapy is able to relieve the severity of AP, it is difficult to block each pathway due to the complicated network of cytokines[2]. Therefore, knowing how to inhibit the initial inflammatory reaction is the key to the treatment of AP. It was reported that inflammatory reaction is correlated to the death modes of pancreatic acinar cells[3]. If death of pancreatic acinar cells occurs in the mode of apoptosis, the cell membrane is intact and there is no release of inflammatory mediators and pancreatin, the inflammatory reaction may be mild[4]. However, if death of pancreatic acinar cells occurs in the mode of oncosis, various pancreatin and inflammatory mediators may release, thus causing a variety of inflammatory cell aggregations and inducing intense inflammatory reactions[5]. If we can induce apoptosis and reduce oncosis, intense inflammatory reactions may be inhibited.

In this study, apoptosis of pancreatic acinar cells was induced by the apoptosis inductor-artemisinin. Changes in apoptosis, oncosis and secondary inflammatory reaction were observed.

MATERIALS AND METHODS

Experimental protocol

Twenty-four male Wistar rats (200 g ± 20 g) were provided by the Animal Research Center of the First Clinical College of Harbin Medical University (Harbin, China), and divided into 3 groups (8 rats in each group): control group, AP group, and artemisinin-treated group (apoptosis inducing group). AP was induced by 4 intraperitoneal injections of caerulein (20 μg/kg, Sigma, USA) at 1 h intervals. To induce apoptosis, solution of artemisinin (2 mg/kg, Huaxin, Sichuan, China) was given intraperitoneally 1, 12, 24 and 36 h after the last caerulein injection. The control rats were only given saline solution. Forty-eight hours after the final injection of caerulein, rats were anaesthetized with sodium pentobarbital (40 mg/kg), and then a laparotomy was performed with the pancreas rapidly removed for further analyses.

Histological examination of pancreatic tissue

Samples of pancreatic tissue were fixed in 20% formaldehyde and processed for paraffin histology. After staining with hematoxylin and eosin (HE), histological grading of interlobular edema, inflammatory infiltration, parenchyma hemorrhage, parenchyma necrosis and vacuolization was valued as previously described[6], then a pathologic score was calculated based on these light microscopic examinations.

Measurement of serum amylase

Serum was collected from the rats for amylase measurement. Amylase level was determined using a commercial chromatometric kit (Jiancheng, Nanjing, China).

Detection of apoptosis and oncosis with acridine orange (AO) and ethylene dibromide (EB) staining

Pancreatic acinar cells were isolated from Wistar rats by two-step collagenase digestion[7] and loaded onto slides with AO (10 μg/mL, Sigma, USA) and EB (10 μg/mL, Sigma, USA) for 10 min. The slides were scanned under confocal laser microscope (Zeiss, Germany) and 500 cells were counted under fluorescent microscope (Nikon, Japan).

Measurement of caspase-3 activity by colorimetric assay

Caspase-3 activity was measured using a colorimetric assay kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. After isolation by two-step collagenase digestion, pancreatic acinar cells were mixed with 50 μL lysis buffer, the supernatant was mixed with 5 μL caspase substrate and 50 μL reaction buffer, and incubated at 37℃ for 4 h in the dark. Fluorescence intensity of the caspase substrate was measured photometrically at 405 nm.

Detection of NF-κB by flow cytometry

Fresh pancreatic tissues were sheared into pieces of 1.0 mm^3 with scissors, and centrifuged at 200 × *g* for 5 min. Then 10 mL detergent solution containing 1% TritonX-100 (Sigma,USA) was added, and stored in a refrigerator at 4℃ for 18 h, then filtered through 50 μm nylon meshes. For fluorescent labeling, 1 μL RNAase was added into 50 μL nucleus suspension and water-bath at 37℃ for 30 min, then 40 μL NF-κB p65 monoclonal antibody (Santa Cruz, USA) was added and incubated at room temperature for 20 min. The samples were treated with 1 μL FITC-labeled second antibody (Jackson Immuno Research, USA) and incubated at room temperature for another 20 min. After treated with 20 μL propidium iodide (PI, Sigma, USA) for 30 min in a dark room, the samples were analyzed using a FACScan flow cytometer (Becton Dicknson, USA).

Measurement of macrophage inflammatory protein-1α (MIP-1α) by Western blot

MIP-1α was detected by Western blot analysis, total protein extract was separated by 10% SDS/PAGE before it was transferred electrophoretically (100 V, 1 h) to hybond C membrane. The membranes were probed with 10 μL anti-MIP-1α monoclonal antibody (Santa Cruz, USA) in TBS-T (1:500), and the immunocomplexed membranes were re-probed at room temperature for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Jackson Immuno Research, USA) in TBS-T (1:500), with 5% blocking reagent. At last, the immunoreactive proteins were visualized using the ECL Western blot analysis system (Amersham, UK).

Chromatometric detection of myeloperoxidase (MPO) activity

The pancreatic tissue was frozen in liquid nitrogen and homogenated. MPO activity was detected with a chromatometric kit (Jianchen, Nanjing, China) following the manufacturer's instructions, and data were expressed as the change in absorbance at 460 nm.

Detection of interleukin-1β (IL-1β) mRNA by semi-quantitive RT-PCR

Total RNA was extracted from pancreatic tissue using Trizol reagent (Invitrogen, USA) and reversely transcriped into cDNA according to the instructions of the kit (Promega, USA). The resulting cDNA was used as a template for subsequent polymerase chain reaction (PCR). The sequences of rat-specific primers for IL-1β (519 bp) are as follows: 5'CCAGGATGAGGACCCAAGCA3' (sense), 5'TCCCGACCATTGCTGTTTCC3' (antisense). Housekeeping gene β-actin (348 bp) was used as a controller, 5'CATCACCATTGGCAATGAGCG3' (sense), 5'CTAGAAGCATTTGCGGTCGGAC3' (antisense). The PCR products were resolved in 1.0% agarose gel for electrophoresis and photographed under ultraviolet transillumination and the intensity of PCR products was measured using a video image analysis system.

Statistical analysis

Data were expressed as mean ± SD. Differences between the groups were tested for significance by one-way analysis of variance (ANOVA), and intergroup comparison was made by Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

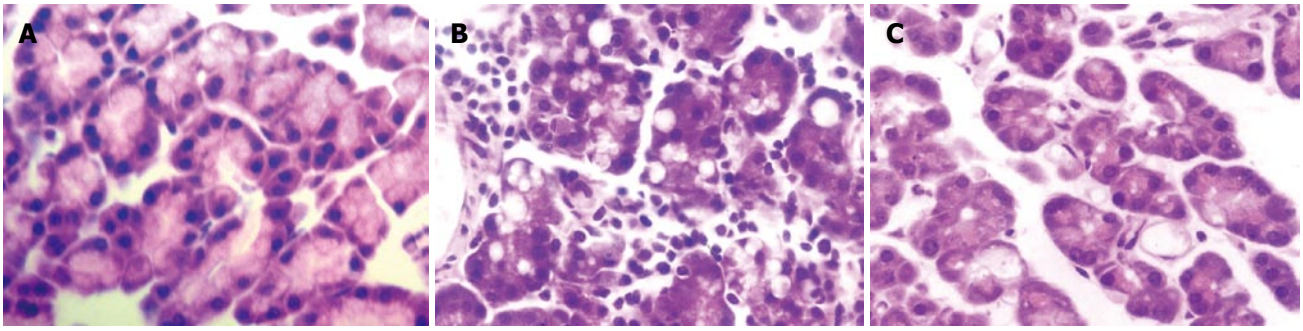

Figure 1 Representative HE-stained pancreatic tissue from the rats (original magnification × 40) in the control group (**A**), AP group (**B**) and artemisinin-treated group (**C**).

Table 1 Detection of cell death pathway and pathologic changes in different groups (mean ± SD, n = 8)

	Pathological score	Amylase (U/dL)	Apoptosis index (%)	Oncosis index (%)	Caspase-3 activity
Control	0.25 ± 0.03	2887 ± 298	1.5 ± 0.3	2.1 ± 0.3	0.35 ± 0.04
AP	4.0 ± 0.5[a]	7821 ± 653[a]	6.3 ± 0.7[a]	1.03 ± 0.08[a]	17.5 ± 2.2[a]
Artemisinin	3.0 ± 0.5[c]	5642 ± 721[c]	11.7 ± 1.4[c]	13.0 ± 2.4[c]	1.52 ± 0.21[c]

AP: Acute pancreatitis. [a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* AP group.

RESULTS

Histological changes after induction of apoptosis

Pancreatic tissue was normal in the control group with a low pathological score. In the AP group, pancreatic tissue displayed lobular mesenchymal rarefaction, edema and inflammatory cell infiltration. In contrast, in the artemisinin-treated group, edema and inflammatory cell infiltration were significantly relieved compared with the AP group (Figure 1). The pathological score showed alleviated pathological impairment in pancreatic tissue after treated with artemisinin ($P < 0.05$ *vs* the AP group) (Table 1).

Effect of artemisinin on induction of apoptosis and avoidance of oncosis

As shown in Figure 2, the nuclei of normal cells showed normal morphology of green fluorescence, while apoptotic cells showed shrunk, condensed or splitted nuclei (green). EB could be resisted by the intact cytoplasmic membrane of normal and apoptotic cells. EB could penetrate the cytoplasmic membrane of oncotic cells, and stain the nuclei of orange-stained cells. So, the apoptotic index or oncotic index, i.e., the number of apoptotic cells or oncotic cells per 100 cells, could be calculated (Figure 3). The results indicate that only sporadic apoptotic or oncotic cells were observed in the control group, but more in the AP and artemisinin-treated groups. In the AP group, there were less apoptotic cells and more oncotic cells. The number of apoptotic cells increased and the number of oncotic cells decreased significantly in the artemisinin-treated group ($P < 0.05$) (Table 1).

Effect of artemisinin on caspase-3 activity

The activity of caspase-3 in isolated pancreatic acinar cells was low in the control group, and high in the AP group, which was significantly elevated after apoptosis was induced by artemisinin ($P < 0.05$) (Table 1).

Influence of apoptosis on NF-κB activation

Activation of NF-κB in normal pancreatic nuclei was significantly higher in the AP group than in the control group ($P < 0.05$) (Figure 4, Table 2).

Influence of apoptosis on MIP-1α

MIP-1α level was low in the control group and high in the AP group. It was obviously lower in the artemisinin-treated group than in the AP group ($P < 0.05$) (Figure 5, Table 2).

Influence of apoptosis on IL-1β mRNA expression

The expression of IL-1β mRNA was low in the control group and high in the AP group. It was lower in the artemisinin-treated group than in the AP group ($P < 0.05$) (Figure 5, Table 2).

Changes in MPO contents after induction of apoptosis

Since each neutrophil granulocyte contains a certain quantity of MPO, detection of MPO in pancreatic tissue reflects the degree of neutrophil infiltration of the tissue. In our study, the MPO concentration was low in the control group and high in the AP group. However, it was down-regulated after induction of apoptosis ($P < 0.05$) (Table 2).

DISCUSSION

AP characterized not only by pancreas impairment, but also by inflammatory cell infiltration and release of various kinds of inflammatory mediators, can develop into SIRS and MODS in some cases and endanger their life[8,9]. Initiation of inflammatory reaction is related to the death pathway of impaired pancreatic acinar cells[10]. Since the conception of apoptosis was proposed by Kerr in 1972[11], apoptosis has been extensively studied. In recent years, attention has been paid to another cell death pathway-oncosis, and it was gradually realized that oncosis makes no less sense than apoptosis[12]. Oncosis has a feature of cell swelling, and cell membrane integrity is destroyed and DNA is split into non-specific fragments. Finally the cells are dissolved accompanying inflammatory reaction. In some physiological and pathological processes, these two kinds of death pathways exist simultaneously, and may

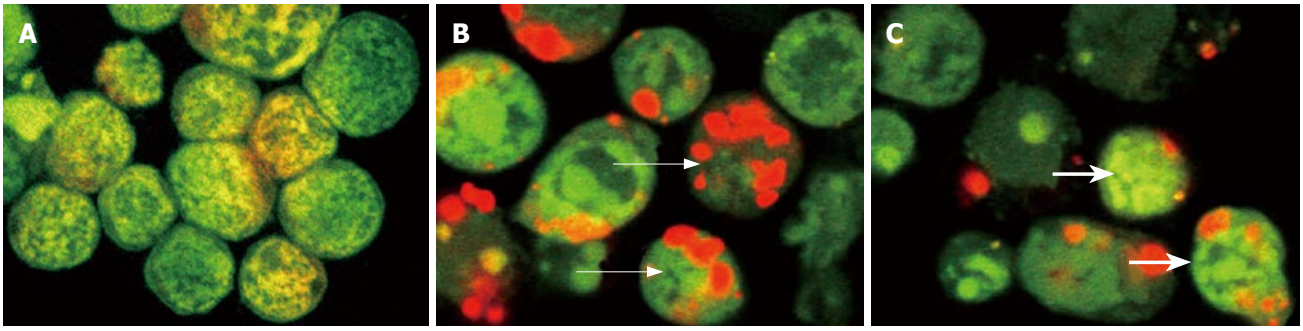

Figure 2 Isolated pancreatic acinar cells stained with AO and EB, and scanned under confocal laser microscope (original magnification × 100) in the control group (**A**), AP group (**B**), and artemisinin-treated group (**C**). Fine arrows indicate oncotic cells, and thick arrows indicate apoptotic cells.

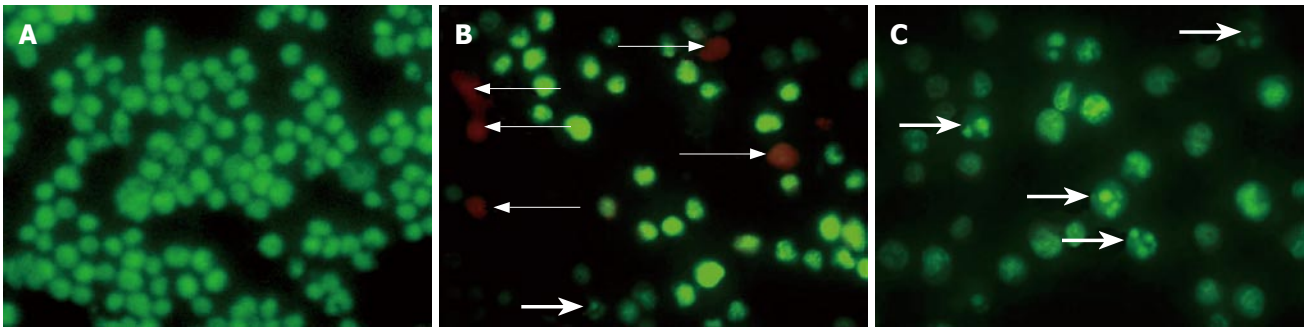

Figure 3 Isolated pancreatic acinar cells stained with AO and EB, and observed under fluorescent microscope (original magnification × 40) in the control group (**A**), AP group (**B**), and artemisinin-treated group (**C**). Fine arrows indicate oncotic cells, and thick arrows indicate apoptotic cells.

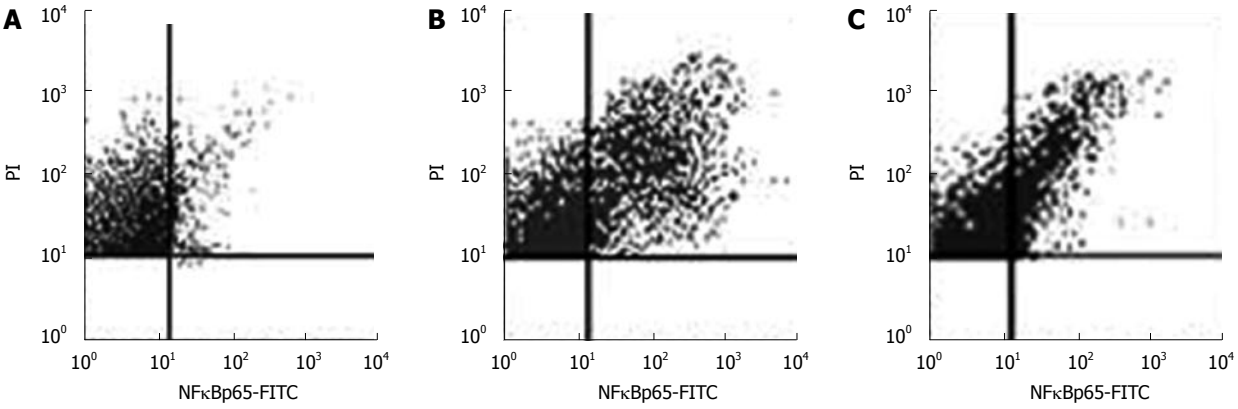

Figure 4 Flow cytometric analysis of NF-κB activation in the control group (**A**), AP group (**B**), and artemisinin-treated group (**C**).

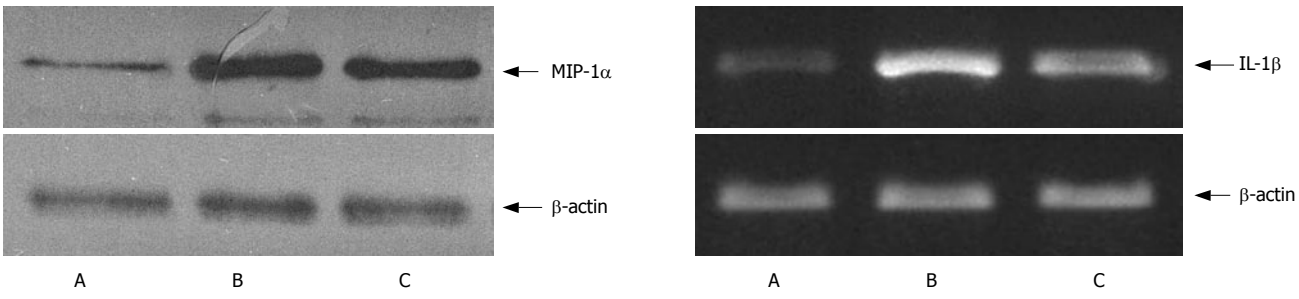

Figure 5 MIP-1α measured by Western blot and IL-1β mRNA detected by RT-PCR in the control group (A), AP group (B), and artemisinin -treated group (C).

Table 2 Inflammatory response of pancreatic tissue in different groups (mean ± SD, n = 8)

	NF-κB activation (%)	MIP-1α protein	MPO (U/g)	IL-1β mRNA
Control	4.7 ± 0.6	0.9 ± 0.1	0.19 ± 0.03	0.5 ± 0.1
AP	42 ± 5.8[a]	5.8 ± 0.7[a]	0.68 ± 0.09[a]	2.4 ± 0.4[a]
Artemisinin	29 ± 4.1[c]	3.7 ± 0.5[c]	0.52 ± 0.06[c]	1.7 ± 0.3[c]

MIP: macrophage inflammatory protein; MPO: myeloperoxidase. [a]P < 0.05 *vs* control group; [c]P < 0.05 *vs* AP group.

interconvert to each other under certain conditions[13]. Oncotic cells may release much more entocytes (including digestive enzyme and inflammatory active medium). It not only destroys the local tissue, but also activates mononuclear cells, leading to SIRS. However, apoptosis may not cause secondary inflammation. During AP, the patient has his or her own self-regulating mechanism. Under certain conditions, the apoptotic signal conduction pathway can be initiated, and more apoptosis will be induced and harmful effects of oncosis may be relieved. However, if AP develops quickly or the patients' self-regulation is in disorder, the apoptosis signal conduction pathway cannot be initiated in time, causing predominant oncosis, leading to aggravation of the disease. This has been proved by Kaiser *et al*[14], who found that when apoptosis is inhibited by cycloheximide, AP obviously aggravates. However, Bhatia *et al*[15] induced apoptosis during their experiment, resulting in the relief of AP. It was reported that one of the therapeutic mechanisms of somatostatin analogue, the most effective drug for AP, is to induce apoptosis of impaired pancreatic acinar cells[16]. Whether AP can be controlled by inducing apoptosis of pancreatic acinar cells has been extensively studied.

Artemisinin is an important active component of traditional Chinese medicine which can induce apoptosis[17,18]. Hahm *et al*[19] found that apoptotic index is elevated but pathological changes in AP rats treated with DA-9610 (an extract from Artemisia Asiatica). In this study, artemisinin was used as an apoptosis inductor. After artemisinin was added into pancreatic acinar cells stimulated by caerulein, apoptosis and oncosis were detected with AO and EB staining, the number of apoptotic cells increased, but the number of oncotic cells decreased. Caspase-3 is a key molecule in the process of apoptosis. Generally, the precursor of caspase-3 in cells is incompetent. However, if it is activated, it can cleave important structural and functional proteins inside the cells and cause chromosome condensation, DNA fragmentation, nuclear membrane rupture, *etc*, finally resulting in apoptosis. The presence of activated caspase-3 indicates that apoptosis is at the irreversible stage[20]. Nam *et al*[21] found that artemisinin can induce apoptosis by up-regulating caspase-3. Our study also showed that the activity of caspase-3 in pancreatic acinar cells was obviously elevated after artemisinin was added, indicating that artemisinin may promote apoptosis. We examined the pancreatic tissue with HE staining and found that after induction of apoptosis, infiltration of inflammatory cells decreased and the pancreas impairment was relieved. Based on this understanding, we observed the degree of inflammatory reaction after induction of apoptosis.

We detected transcription factor-NF-κB which regulates synthesis of many inflammatory mediators and cytokines[22]. NF-κB is a protein that regulates gene transcription, participates in regulating many inflammatory factors, and evokes immune and inflammatory reactions[23]. NF-κB plays a key role in the development of AP[24]. In our study, activation of NF-κB in the apoptosis inducing group was obviously decreased, compared with the AP group, indicating that activation of NF-κB can be decreased by inducing apoptosis and reducing oncosis. The conserved sequence of MIP-1α combined with NF-κB exists in its promoter region[25], suggests that MIP-1α may be one of the downstream targets regulated by NF-κB. MIP-1α was detected by Western blot assay in this study, proving that if apoptosis is induced, MIP-1α can be inhibited. MIP-1α is a CC-type chemotatic factor and plays an important role in recruiting mononuclear cells and lymphocytes[26]. Just as the "over-activation of leucocyte theory" proposed by Rindernech *et al*[27], AP aggravates because inflammatory cells are over-activated, and these activated cells such as granulocytes and macrophages, play a great role in the development of AP. Therefore, MPO (the marker of neutrophils) and IL-1β (the inflammatory factors generated mainly by mononuclear macrophages)[28] were detected in this study, indicating that MPO is obviously decreased in pancreas tissue after induction of apoptosis, reducing neutrophil recruition and infiltration to pancreas tissue. IL-1β is a kind of proinflammatory cytokines mainly generated by macrophages in pancreas when AP occurs. IL-1β can activate neutrophils, up-regulate the expression of surface adhesion molecules of lymphocyte and endotheliocytes[29]. Fink *et al*[30] reported that release of pancreatic amylase and necrosis of pancreas tissue are obviously decreased by blocking IL-1 receptor, demonstrating that IL-1 is essential to inflammation and development of AP. Our study proved that after induction of apoptosis, the level of IL-1β mRNA in pancreas tissue was low, indicating that infiltration and activation of macrophages are decreased and inflammatory reaction is inhibited after induction of apoptosis.

In conclusion, infiltration of inflammatory cells and generation of inflammatory cytokines can be decreased by inducing apoptosis and reducing oncosis of pancreatic acinar cells.

COMMENTS

Background

One of the greatest findings in AP is the initiation of cytokine network in AP patients that promotes occurrence of SIRS and MODS. Efforts have been made to alleviate pathological changes in AP by inhibiting the cytokine network. Since cytokine network is so complex that it is impossible to block all the pathways, it may pave a new way for the treatment of AP to obstruct the cytokine chain reactions. It was reported that the cytokine network can be obstructed by inducing apoptosis, decreasing oncosis and release of endocellular enzyme, suggesting that any drugs regulating apoptosis may be used in the treatment of AP.

Research frontiers

It has been verified that AP worsens when apoptosis of pancreatic acinar cells is

inhibited by cycloheximide, and that AP is alleviated when apoptosis is induced. It was reported that the severity of AP is associated with the degree of oncosis. Hahm *et al* found that there is an elevated apoptotic index but extenuated pathological changes in AP rats after treated with DA-9610 (an extract from Artemisia Asiatica), suggesting that artemisinin can regulate cell death and can be used in the treatment of AP.

Innovations and breakthroughs

When the concept of apoptosis was first put forward by Kerr in 1972, a lot of studies have been performed with it. In recent years, more and more attention has been paid to oncosis, showing that the importance of oncosis is no less than that of apoptosis. In traditional Chinese medicine, some important prescriptions have been successfully applied in AP treatment, and one of the mechanisms is to suppress inflammatory response and induce apoptosis. Artemisinin is an important active component of traditional Chinese medicine which can induce apoptosis. In this study, we observed the regulatory effect of artemisinin on apoptosis and oncosis of pancreatic acinar cells and its therapeutic effect on AP.

Applications

Artemisinin can induce apoptosis and reduce oncosis of pancreatic acinar cells at the onset of AP, thus repressing the intense inflammatory reactions, such as SIRS and MODS. Therefore, there is a bright prospect for artemisinin in the treatment of AP.

Terminology

Oncosis, or cellular swelling, is a pathological process of cell death, in which the completeness of cell membrane is destructed and DNA is split to non-specific fragments, ultimately leading to cell lysis complicated by inflammatory reactions.

Peer review

The effect of artemisinin on acute pancreatitis was analyzed. The data presented are interesting.

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CLINICAL RESEARCH

Rational prescription of drugs within similar therapeutic or structural class for gastrointestinal disease treatment: Drug metabolism and its related interactions

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Abstract

AIM: To review and summarize drug metabolism and its related interactions in prescribing drugs within the similar therapeutic or structural class for gastrointestinal disease treatment so as to promote rational use of medicines in clinical practice.

METHODS: Relevant literature was identified by performing MEDLINE/Pubmed searches covering the period from 1988 to 2006.

RESULTS: Seven classes of drugs were chosen, including gastric proton pump inhibitors, histamine H_2-receptor antagonists, benzamide-type gastroprokinetic agents, selective 5-HT_3 receptor antagonists, fluoroquinolones, macrolide antibiotics and azole antifungals. They showed significant differences in metabolic profile (i.e., the fraction of drug metabolized by cytochrome P450 (CYP), CYP reaction phenotype, impact of CYP genotype on interindividual pharmacokinetics variability and CYP-mediated drug-drug interaction potential). Many events of severe adverse drug reactions and treatment failures were closely related to the ignorance of the above issues.

CONCLUSION: Clinicians should acquaint themselves with what kind of drug has less interpatient variability in clearance and whether to perform CYP genotyping prior to initiation of therapy. The relevant CYP knowledge helps clinicians to enhance the management of patients with gastrointestinal disease who may require treatment with polytherapeutic regimens.

Key words: Cytochrome P450; Pharmacokinetics; Drug metabolism; Genotype; Polymorphism; Drug interaction; Pharmacotherapy; Gastrointestinal diseases

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INTRODUCTION

More and more drugs within the similar therapeutic or structural class are emerging and it is essential to compare the alternative drug choices according to their efficacy, safety, suitability and cost. However, irrational prescription is common in many countries. Drug metabolism and its related interactions are most prone to be ignored in clinical practice. Actually, metabolism by cytochrome P450 (CYP) represents an important clearance mechanism for the majority of drugs, thus affecting their oral bioavailability, duration and intensity of pharmacological action[1]. The metabolic profile of a drug depicts its amount metabolized by CYP, CYP reaction phenotype, impact of CYP genotype on interindividual pharmacokinetics (PK) variability and CYP-mediated drug-drug interaction potential. It is closely related to the three-dimensional chemical structure of drug and may exhibit significant differences among drugs within the similar therapeutic or structural class, although the efficacy of these similar drugs do not show sharp differences at the dose used clinically[2,3]. The voluntary market withdrawal of cerivastatin by Bayer and withdrawal of medications such as terfenadine, astemizole, cisapride, and mibefradil from the market by the Food and Drug Administration (FDA) further demonstrate the relevance of metabolic drug-drug interaction profile. Although the FDA has published guidance for *in vitro* and *in vivo* drug metabolism/drug interaction

studies in the drug development process[4,5], systematic summary is not yet available on metabolic differences in market products within the similar therapeutic or structural class. This review focuses on seven classes of drugs for gastrointestinal diseases treatment and aims to help clinicians realize what kind of drug has less interpatient variability in clearance, whether to perform CYP genotyping prior to the initiation of therapy, and how to enhance the management of patients on polytherapy regimens.

MATERIALS AND METHODS

Seven classes of drugs for gastrointestinal diseases treatment were chosen, including gastric proton pump inhibitors, histamine H_2-receptor antagonists, benzamide-type gastroprokinetic agents, selective 5-HT_3 receptor antagonists, fluoroquinolones, macrolide antibiotics and azole antifungals. Relevant literature, focusing on drug metabolism, metabolic interaction potentials and clinical events of adverse drug reactions and treatment failures caused by drug-drug interaction, was identified by performing MEDLINE/Pubmed searches covering the period from 1988 to 2006.

RESULTS

Gastric proton pump inhibitors

Proton pump inhibitors (or "PPI"s) are a group of drugs widely prescribed for the treatment of acid-related diseases such as peptic ulcer, gastroesophageal reflux disease (GERD), nonsteroidal anti-inflammatory drug induced gastropathy and Zollinger-Ellison syndrome. Currently used PPIs in clinical practice are as follows: omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole. All are benzimidazole derivatives (Figure 1). Schematic depiction of metabolic differences among four PPIs is described in Figure 2.

Lansoprazole, omeprazole and pantoprazole are all primarily metabolized by CYP2C19, an isoenzyme that exhibits genetic polymorphism with 15%-20% of Asian populations being poor/slow metabolizers, whereas the prevalence is much lower (3%-5%) among Caucasians and Blacks[6]. Their PK behaviors are all dependent on CYP2C19 genotype. $AUC_{po(PM)}/AUC_{po(EM)}$, the ratio of parent drug area-under-the concentration *vs* time curve after oral dosing (AUC_{po}) derived from poor metabolizers (PM) and extensive metabolizers (EM), is 7.4, 3.7 and 6.0 for omeprazole, lansoprazole and pantoprazole, respectively[7]. CYP2C19 polymorphism is also a major predictor of treatment failures in patients receiving lansoprazole-, omeprazole- or pantoprazole based polytherapy for eradication of *H pylori*[8].

Omeprazole has also been known as a potent inhibitor of CYP2C19, and may cause pharmacokinetic interactions with other CYP2C19 substrates such as diazepam, phenytoin and moclobemide[9-11]. Both lansoprazole and omeprazole also induce CYP1A2 *in vitro*[12]. Omeprazole can reduce clozapine plasma concentrations by 40%[13]. However, concomitant intake of omeprazole or lansoprazole at high therapeutic doses does not affect the PK behavior of theophylline and caffeine[14,15]. The underlying explanation of the discrepancy may be that inducibility of CYP1A2 by

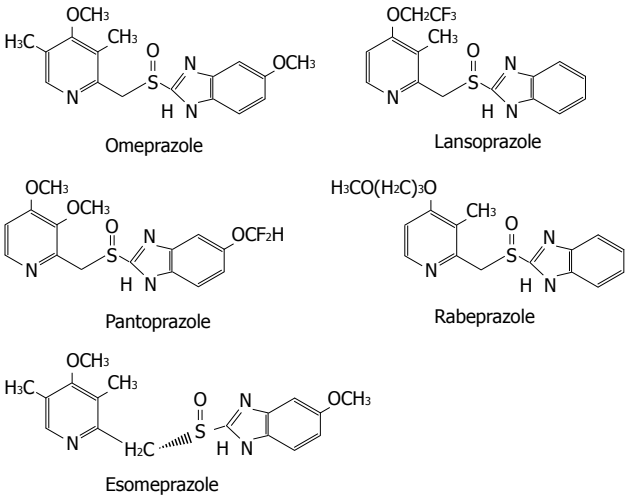

Figure 1 Chemical structures of five PPIs.

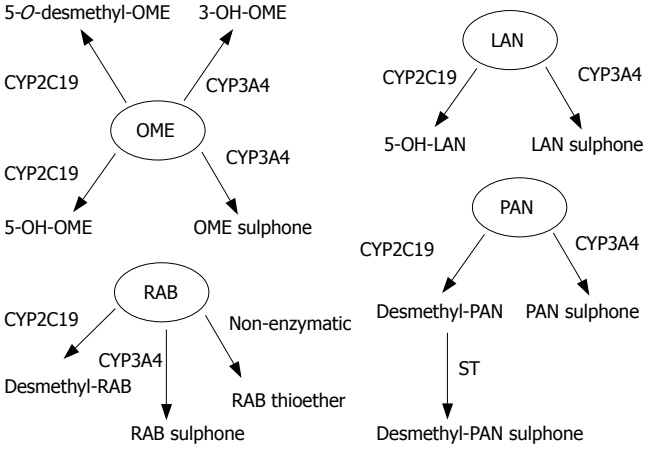

Figure 2 Metabolic differences between four PPIs (OME: omeprazole; LAN: lansoprazole; RAB: rabeprazole; PAN: Pantoprazole). Arrow thickness represents relative contribution to metabolism.

omeprazole *in vivo* is related to the genetic polymorphism of CYP1A2, dose and course of treatment[16-18]. Potential interactions between omeprazole or lansoprazole and CYP1A2 substrates with narrow therapeutic windows should be kept in mind in long-term concurrent therapy. Among these three old PPIs, pantoprazole has by far the lowest potential for interactions[19].

Rabeprazole, although metabolized partially by CYP2C19, is primarily metabolized by nonenzymatic reduction and hence genotype and modifiers of CYP2C19 have less impacts on its PK ($AUC_{po(PM)}/AUC_{po(EM)} \leqslant 1.8$) and clinical efficacy[20].

Esomeprazole is the S-enantiomer of omeprazole. Its metabolism involves CYP2C19, but to a lesser extent than omeprazole (Figure 3). Its PK is less dependent on CYP2C19 genotype ($AUC_{po(PM)}/AUC_{po(EM)}$ approximate 3.0) and hence, it has less interpatient variability in clearance than omeprazole. Moreover, esomeprazole is cleared more slowly *in vivo* and has an improved oral bioavailability, leading to the greater inhibition of gastric acid secretion compared to omeprazole[21,22].

The enantiomers of pantoprazole are differentially

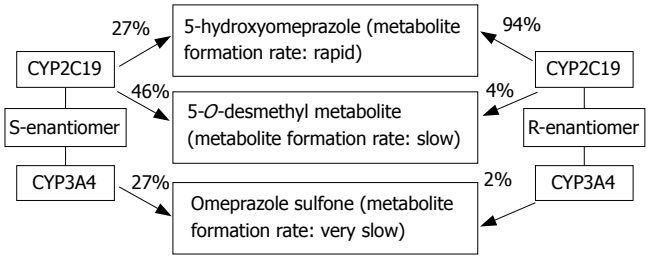

Figure 3 Stereoselective metabolism of omprazole in human.

affected by CYP2C19 genotype, such that the $AUC_{po(PM)}$/$AUC_{po(EM)}$ ratio is 11 and 2.5 for the R-(+)- and S-(−)-enantiomers, respectively[23]. Comparative clinical trial of S-(−)-pantoprazole *vs* racemic pantoprazole in the treatment of GERD has been carried out by Pai *et al*[24]. S-(−)-pantoprazole (20 mg) was found to be more effective than racemic pantoprazole (40 mg) in improving symptoms. Consequently, the use of S-(−)-pantoprazole offers both pharmacokinetic and pharmacodynamic advantages.

Many recent cost-effectiveness analyses have provided an economic basis to employ CYP2C19 genotyping prior to initiating omeprazole-, lansoprazole- or pantoprazole-based polytherapy. However, pharmacogenetic tests may be unnecessary if rabeprazole or esomeprazole based therapy are considered.

Histamine H_2-receptor antagonists

Histamine H_2-receptor antagonists are clinically applied for the treatment of gastritis, gastric and duodenal ulcers[25]. Six H_2-receptor antagonists are currently on the market: cimetidine, ranitidine, famotidine, nizatidine, ebrotidine and roxatidine acetate. Their chemical structures are depicted in Figure 4.

Martinez *et al*[26] compared the inhibitory effect of the H_2-receptor antagonists on the enzymes activities in human liver microsomes. The results were as follows: CYP1A2: cimetidine > ranitidine = ebrotidine; CYP2D6: cimetidine > ranitidine = ebrotidine; CYP3A4: ebrotidine > cimetidine > ranitidine. However, it should be cautiously considered when these *in vitro* data were extrapolated to *in vivo* situations. Firstly, cimetidine only selectively inhibits *in vivo* activities of CYP3A4 and CYP2D6[27]. For example, coadministered cimetidine increased the degree of beta-blockade of timolol (CYP2D6 substrate) ophthalmic solution and the maximum plasma concentrations of CYP3A4 substrates (e.g., midazolam and saquinavir) and disopyramide (CYP3A4 and CYP2D6 substrate)[26,28-30]. Coadministration of cimetidine 400 mg twice a day with saquinavir soft gel 1200 mg twice a day resulted in a significant increase in saquinavir $AUC_{0\text{-}24}$ (120%) and C_{max} (179%). From this view, coadministered cimetidine may be employed as a new pharmacoenhancer for boosting saquinavir for HIV infections. Beneficial effects of the inhibitory activity of cimetidine toward CYP are also used for the prevention of hepatotoxicity induced by overdoses with paracetamol, a substrate of several CYP isoenzymes which activate the drug by oxidation to the hepatotoxic metabolite N-acetyl-p-benzoquinone imine.

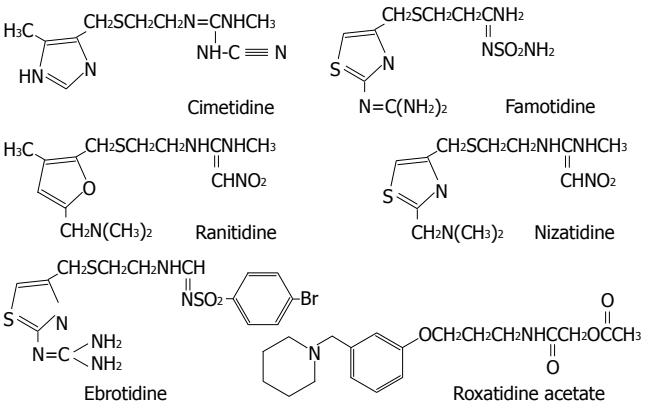

Figure 4 Chemical structures of six H_2-receptor antagonists.

Secondly, inhibition of CYP1A2 activity in humans by cimetidine has not been observed with clinical significance. In concurrent therapy of warfarin, disposition of the less potent R-warfarin (CYP1A2 substrate) was impaired. However, this interaction is likely to be of minimal clinical significance in most patients[31]. The interaction between cimetidine and theophylline was reported inconsistently. Degree of inhibition (absolute change in theophylline clearance) was closely related to route of administration, dosage, the basal theophylline clearance and smoking history[32-35]. Significant pharmacokinetic interaction between cimetidine and theophylline was not observed with low-dose cimetidine (200 mg twice daily), but with 800 mg cimetidine given once daily. Smokers or individuals with higher basal theophylline clearances had greater degree and percent of inhibition than non-smokers or individuals with lower basal theophylline clearances. It suggests that disposition of CYP1A2 substrates may still be impaired in smokers or other individuals with high CYP1A2 activities when coadministered with cimetidine. Thirdly, ebrotidine has no inhibitory effect on CYP3A4 *in vivo*, which is confirmed by lack of metabolic interaction with midazolam[26]. Overall, in contrast to cimetidine, the effects of the other H_2-receptor antagonists on CYP *in vivo* seem to have little clinical significance.

Benzamide-type gastroprokinetic agents

Benzamide-type gastroprokinetic agents (e.g., metoclopramide, cisapride, mosapride, itopride, renzapride and domperidone) are the mainstay of therapy in disorders of gastric motility such as non-ulcer dyspepsia (NUD), GERD, gastritis, diabetic gastroparesis and functional dyspepsia. Their chemical structures are depicted in Figure 5.

Among these gastroprokinetic agents, metoclopramide is predominantly metabolized by CYP2D6, thus its elimination being slow in PMs of CYP2D6 or in patients taking inhibitors of this isoform. Metoclopramide-induced acute dystonic reactions were more frequently observed in patients carrying homozygous CYP2D6 polymorphisms[36]. Meanwhile, it is also a potent inhibitor of CYP2D6 at therapeutically relevant concentrations and markedly inhibits *in vitro* codeine bioactivation[37,38]. Human pharmacokinetic interactions between metoclopramide and CYP2D6 substrates have not yet been documented.

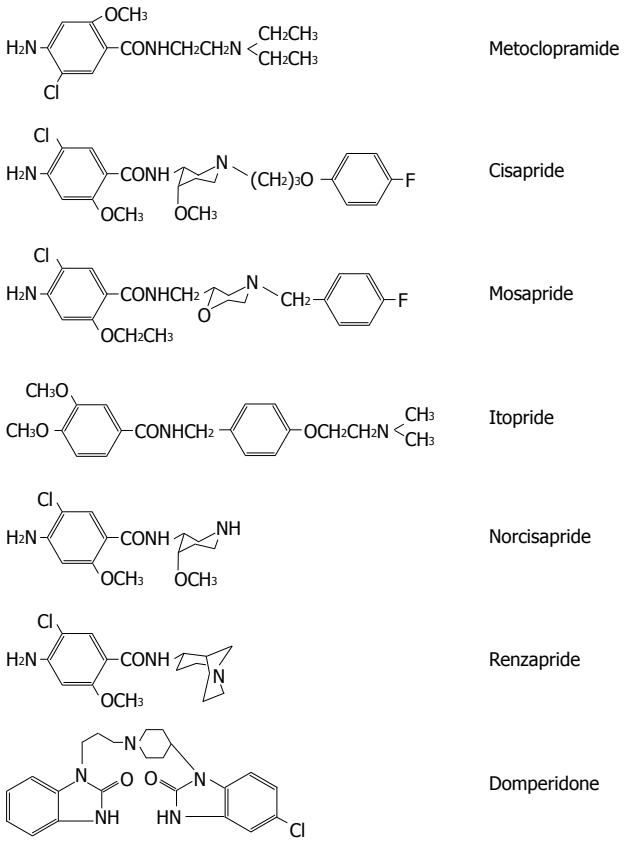

Figure 5 Chemical structures of seven benzamide-type gastroprokinetic agents.

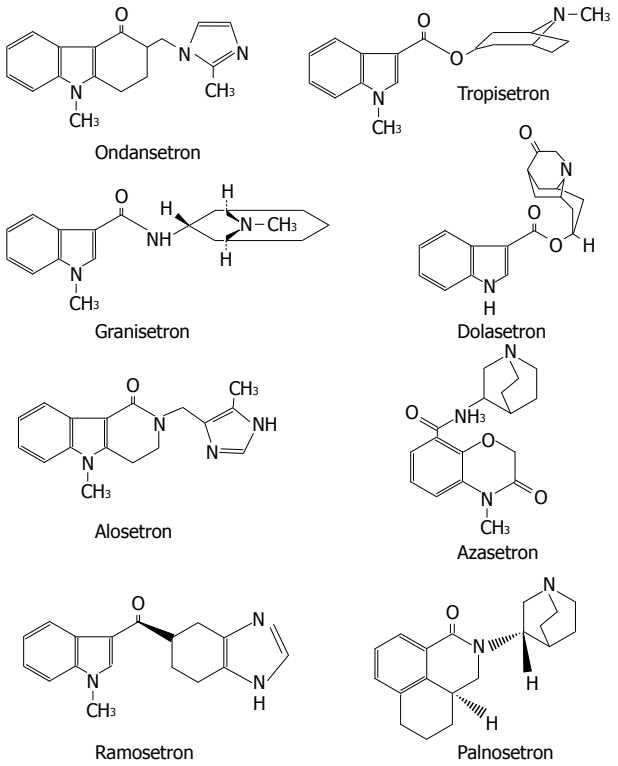

Figure 6 Chemical structures of eight setrons.

Cisapride, mosapride and domperidone, are all predominantly metabolized by CYP3A4. Their dispositions could be strongly impaired by CYP3A4 inhibitors, causing greatly elevated plasma concentrations of parent drugs[39-41]. Among these prokinetic agents, only interactions of cisapride and CYP3A4 inhibitors induce severe clinical adverse events like QT interval prolongation and/or torsades de pointe, which is responsible for the withdrawal of cisapride by FDA. However, cisapride is still on the market in some countries following restriction imposed on its usage. The most important step that can be taken to minimize the risk of cisapride-associated arrhythmias is to avoid the concomitant administration of contraindicated drugs, particularly the macrolide antibiotics (e.g., erythromycin, clarithromycin) and the azole antifungals, (e.g., itraconazole and ketoconazole).

Itopride is primarily metabolized by flavin-containing monooxygenase and its PK is unlikely influenced by CYP3A4 inhibitors[39]. Norcisapride is a major active meta-bolite of cisapride *via* CYP3A4-mediated N-dealkylation. It possesses approximately 15% of the prokinetic activity of cisapride, but has no apparent effect on myocardial conduction[42]. Compared with cisapride, norcisapride elimination does not depend on CYP, and so norcisapride does not interact with azoles or macrolides. Janssen Pharmaceutica has licensed Sepracor's patent on (+) -norcisapride, and its clinical trials are undergoing. This new compound along with mosapride, domperidone and itopride are potentially safer alternatives to cisapride in the concurrent therapy of gastroprokinetic agents with potent CYP3A4 inhibitors.

Renzapride is not metabolized by CYP. It is excreted *via* the renal route primarily unchanged. Thus, its disposition is unsusceptible to CYP modulators and it does not interfere with CYP-mediated metabolism of other drugs[43]. It is currently in clinical development for constipation-predominant irritable bowel syndrome.

Selective 5-HT$_3$ receptor antagonists

The selective 5-HT$_3$-receptor antagonists or "setrons", including ondansetron, dolasetron, tropisetron, granisetron, alosetron, azasetron, palonosetron and ramosetron (Figure 6) represent a class of antiemetics that are currently used for chemotherapy- and radiotherapy-induced, or postoperative nausea and vomiting. However, these setrons have different metabolic profiles.

Ondansetron is cleared by multiple CYP forms in humans, with no single CYP form dominating the overall metabolism. Therefore, its PK lacks bimodality and seems unchanged when ondansetron is used concomitantly with specific CYP isoenzyme inhibitors[44].

Dolasetron is rapidly reduced by carbonyl reductase to its major active metabolite hydrodolasetron, which is eliminated by multiple routes, including renal excretion and metabolism mainly by glucuronidation and hydroxylation[45]. Hence, dolasetron appears to be insusceptible to clinically significant metabolic interactions posed by drugs commonly used in chemotherapy or surgery.

Tropisetron metabolism is almost exclusively CYP2D6-dependent and the metabolites are not pharmacologically active, thus the efficacy of antiemetic treatment with tropisetron largely depends on CYP2D6 genotype. The dose of tropisetron has to be patient-tailored according to CYP2D6 genotype[46,47].

Granisetron is unique because it is not metabolized *via*

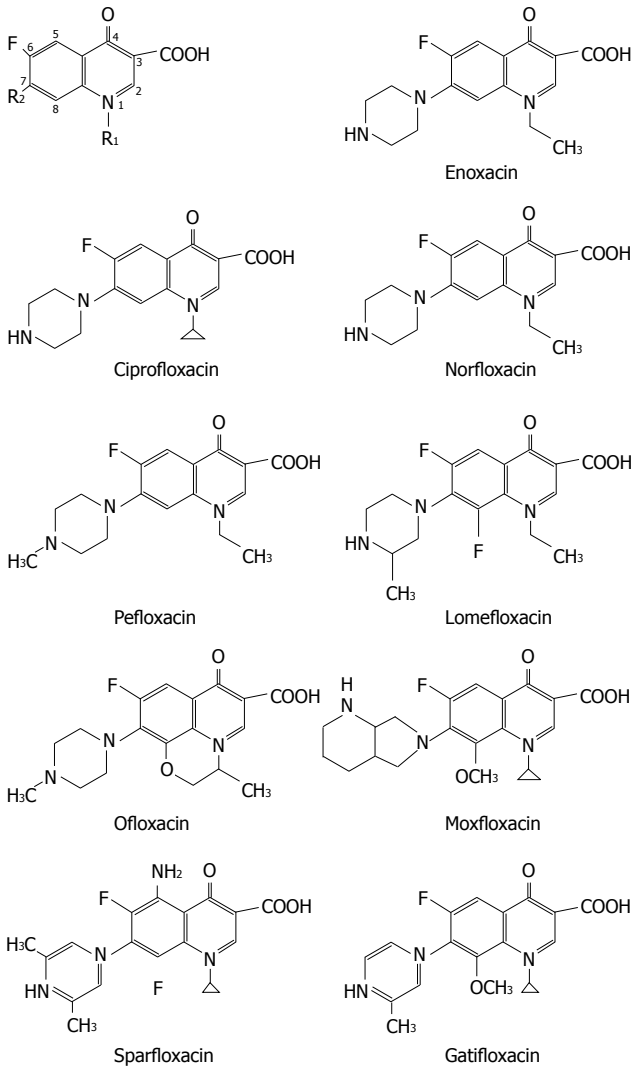

Figure 7 Chemical structures of nine fluoroquinolones.

CYP2D6. Instead, it is metabolized *via* CYP3A4, which is not subject to significant genetic polymorphism and variation in patient response. Moreover, carriers of the duplication of the CYP2D6 allele predicting ultrarapid metabolizer status had less frequent vomiting episodes in subjects receiving granisetron than patients receiving tropisetron. Use of granisetron would obviate the need for CYP2D6 genotyping and may lead to improved prophylaxis of postoperative nausea and vomiting[48-50].

Alosetron is extensively metabolized in humans. *In vivo* data suggest that CYP1A2 plays a prominent role in alosetron metabolism[51,52]. In a pharmacokinetic study, 40 healthy female subjects received fluvoxamine (a known strong inhibitor of CYP1A2) in escalating doses from 50 to 200 mg per day for 16 d, with coadministration of alosetron 1 mg on the last day. Fluvoxamine increased mean alosetron AUC by approximately 6-fold and prolonged the half-life by approximately 3-fold. Thus, concomitant administration of alosetron and strong inhibitor of CYP1A2 is contraindicated. Otherwise, dose-related side effects of alosetron such as constipation may occur more frequently[53].

Azasetron is mainly excreted in urine as the unmetabolized form (approximately 60%-70%), which is different from the fact that other setrons undergo extensive metabolism[54]. *In vitro* data suggest that azasetron does not cause clinically significant CYP-mediated drug interactions.

Palonosetron is metabolized in the liver (approximately 50%). The two primary metabolites, N-oxide-palonosetron and 6-(S)-hydroxy-palonosetron, are essentially inactive. CYP2D6 is the major enzyme of palonosetron metabolism. Clinical pharmacokinetic parameters were not significantly different between PMs and EMs of CYP2D6[55-57]. As for ramosetron, *in vitro* data with human liver microsomes showed its minimal potential to cause clinically important CYP-mediated drug interactions[58].

Fluoroquinolones

Fluoroquinolones are good choices in treatment of intestinal infections caused by sensitive bacterias. Meanwhile, fluoroquinolones-based polytherapy regimens are also used for *H pylori* infection in some occasions, especially after treatment failure in initial *H pylori* eradication[59-63].

The chemical structures of nine fluoroquinolones are listed in Figure 7. They have different CYP-mediated interaction potentials. Enoxacin, ciprofloxacin, norfloxacin and to a lesser extent pefloxacin all have inhibitory effects on metabolism of CYP1A2 substrates such as warfarin, tacrine, clozapine, tizanidine and theophylline. Ofloxacin, levofloxacin, sparfloxacin, lomexacin, gatifloxacin, sparfloxacin, lomefloxacin and moxifloxacin, are less prone to inhibit CYP1A2 and thus are alternative fluoroquinolones to patients receiving concurrent therapy of CYP1A2 substrate with narrow therapeutic window.

Moreover, ciprofloxacin and norfloxacin significantly depressed CYP3A4 in human microsomes[64]. Many case reports indicated their inhibitory effects on CYP3A4 in humans[65-69]. Clinicians should be wary of coadministration of norfloxacin or ciprofloxacin with CYP3A4 substrates with narrow therapeutic window. Table 1 lists the metabolic drug interactions related to fluoroquinolones with clinical relevance.

Macrolide antibiotics

Macrolide antibiotics are usually included in polytherapy regimen for treatment of *H pylori* gastritis[76-80]. In addition, erythromycin, clarithromycin and azithromycin all exhibit prokinetic effects and may be used in the management of gastroparesis[81-84]. For example, erythromycin therapy is effective in the treatment of patients with gastroparesis, in whom metoclopramide or domperidone was ineffective.

Macrolides can be classified into 3 groups based on the propensity of these compounds to interfere with CYP3A4[85-87]. The first group (e.g., troleandomycin, erythromycin and clarithromycin) are potent mechanism-based CYP3A4 inhibitors. Because mechanism based inhibition is an irreversible inhibition where a covalent bond is formed between a metabolite and the active site of the enzyme, destroying the enzyme's activity, so the first group of macrolides could produce drug interactions with clinical relevance. The second group (e.g., flurithromycin, midecamycin, josamycin and roxithromycin) form complexes to a lesser extent and rarely produce drug interactions. The

Table 1 Metabolic drug interactions of fluoroquinolones with clinical relevance

Polytherapy regimen	Clinical consequence	Ref
Ciprofloxacin + tizanidine	Oral ciprofloxacin (500 mg twice daily for 3 d) increased AUC (0-infinity) of tizanidine by 10-fold and Cmax by 7-fold and dangerously potentiates its hypotensive and sedative effects, mainly by inhibiting CYP1A2. Care should be exercised when tizanidine is used concomitantly with ciprofloxacin.	70
Ciprofloxacin + clozapine	Ciprofloxacin (250 mg twice daily for 7 d) can moderately increase serum concentrations of clozapine and N-desmethylclozapine in patients with schizophrenia. A probable mechanism of interaction is an inhibition of CYP1A2 by ciprofloxacin.	71
Ciprofloxacin + theophylline	The interaction between oral ciprofloxacin (500 mg twice daily for 60 h) and theophylline can be clinically significant. Inter-individual variability in the magnitude of interaction can be attributed to inter-individual differences in the level of CYP1A2 expression.	72
Ciprofloxacin + olanzapine	Ciprofloxacin treatment (250 mg twice daily for 3 d) doubled olanzapine concentrations in one patient through the inhibition of CYP1A2.	73
Ciprofloxacin + sildenafil	Ciprofloxacin significantly increased sildenafil bioavailability (above 2-fold) in healthy volunteers, mainly by CYP3A4 inhibition. Dose adjustment of sildenafil is thus necessary.	65
Ciprofloxacin + methadone	Ciprofloxacin inhibited metabolism of methadone *via* CYP1A2 and CYP3A4, and caused profound sedation, confusion, and respiratory depression	66
Ciprofloxacin + cyclosporine	Ciprofloxacin and cyclosporine may be used together safely at the recommended dosage. However, case reports have suggested a possible pharmacokinetic interaction, e.g., ciprofloxacin substantially increased cyclosporine blood levels in a patient with pure red blood cell aplasia. However, levofloxacin therapy (500 mg/d Ⅳ) did not interfere with cyclosporine blood levels and thus it could be a therapeutic alternative.	67, 68
Enoxacin + fluvoxamine	Enoxacin (200 mg/d for 11 d) significantly increased the plasma concentrations at 2, 3 h and the Cmax of fluvoxamine in healthy volunteers. Sleepiness produced by fluvoxamine increased when coadministered with enoxacin.	74
Enoxacin + theophylline	A multidose regimen of enoxacin significantly slowed the clearance of theophylline and elevated theophylline concentrations in serum. The careful monitoring of serum theophylline level and modification of theophylline dosage in patients receiving enoxacin and theophylline were recommended.	75
Norfloxacin + cyclosporine	In pediatric patients undergoing renal transplantation norfloxacin impaired cyclosporine disposition by inhibition of CYP3A4, resulting in cyclosporine dose reduction from 7.4 mg/kg per day to 4.5 mg/kg per day.	69

last group (e.g., azithromycin, dirithromycin and spiramycin) does not inhibit CYP3A4 and are unable to modify the PK behaviors of other compounds.

Metz *et al*[88] reported a potentially significant pharmacokinetic drug interaction between clarithromycin and carbamazepine in two patients with long-standing epilepsy who received omeprazole-clarithromycin therapy for *H pylori* gastritis. In both cases, clarithromycin therapy was temporally related to an increase in serum carbamazepine levels, which returned to the therapeutic range following cessation of clarithromycin therapy. If possible, erythromycin and clarithromycin should be avoided in patients taking CYP3A4 substrates such as atorvastatin, simvastatin, rifabutin, midazolam, cyclosporin, cisapride, pimozide, disopyramide, astemizole, nifedipine and carbamazepine. Azithromycin may be an alternative[89-92]. If clinical judgment suggests erythromycin and clarithromycin should be used, it is necessary to adjust dosage of CYP3A4 substrates with narrow therapeutic window (e.g., decrease the dosage of carbamazepine by 30%-50%), monitor the serum drug levels closely, and warn the patient about the signs and symptoms of toxicity.

Moreover, both erythromycin and clarithromycin are also potent inhibitors of P-glycoprotein and can significantly interfere with the PK behaviors of P-glycoprotein substrate such as digoxin. For example, a case of a clarithromycin-associated digoxin toxicity in a patient with chronic atrial fibrillation and *H pylori* infection was reported by Gooderham *et al*[93].

Azole antifungals

Azole antifungals (i.e., ketoconazole, itraconazole, fluconazole and voriconazole) may be used in treatment for fungus infections in digestive tracts. Their chemical structures are illustrated in Figure 8.

Ketoconazole is extensively metabolized into several inactive metabolites in the liver and the metabolites primarily excreted in bile[94]. Itraconazole is metabolized predominately by CYP3A4. Renal excretion of the parent drug is less than 0.03% of the dose[95]. Fluconazole is mainly excreted in urine as the unmetabolized form (approximately 80%). Accordingly, renal function is the major determinant of fluconazole PK[96]. The concurrent therapy of CYP3A4 inducers (e.g., rifampin and rifabutin) with itraconazole or ketoconazole results in poor antifungal response, thus their coadministrations are not recommended. However, fluconazole PK is less affected by CYP3A4 inducers[97], so fluconazole may be as an alternative for patients receiving comedicated CYP3A4 inducers.

Voriconazole is extensively metabolized by CYP2C19, CYP2C9 and CYP3A4. The major metabolite of voriconazole is the N-oxide, which has negligible antifungal activity. Inducers or inhibitors of these isoenzymes may increase or decrease voriconazole plasma concentrations. Coadministration of voriconazole with rifampicin, carbamazepine and phenobarbital is contraindicated. Allelic polymorphisms of CYP2C19 have been shown to be the most important determinants of the clearance of voriconazole, resulting in two phenotypes: PMs and EMs (both homozygous and heterozygous). Homozygous EMs have a two-fold lower exposure than heterozygous EMs and four-fold lower drug exposure than PMs[98-100]. Coadministration of a potent CYP3A4 inhibitor leads to a higher and prolonged exposure with voriconazole that might increase the risk of ADRs on a short-term

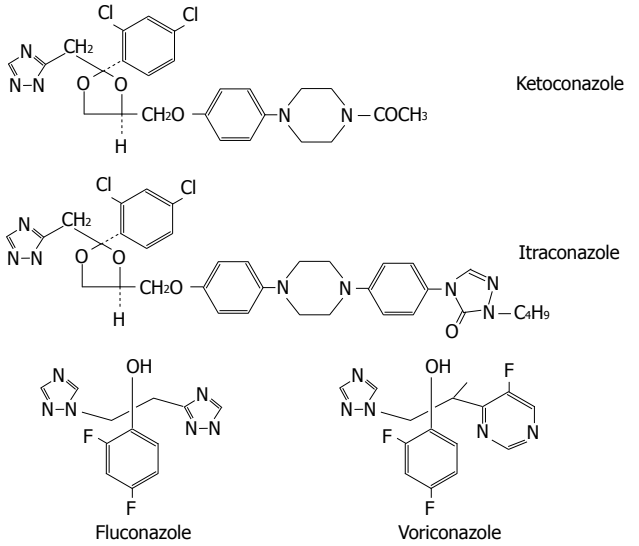

Figure 8 Chemical structures of four azole antifungals.

basis, particularly in CYP2C19 PM patients[101]. Thus, it is necessary to implement CYP2C19 genotyping prior to initiation of voriconazole therapy or therapeutic drug monitoring in the course of treatment.

Ketoconazole and itraconazole are potent inhibitors of CYP3A4. Coadministration with CYP3A4 substrates can cause clinically significant drug interactions, some of which can be life-threatening. Cisapride, oral midazolam, pimozide, quinidine, triazolam, levacetylmethadol, statins metabolized by CYP3A4 (i.e., lovastatin, simvastatin and atorvastatin), ergot alkaloids metabolized by CYP3A4 (i.e., dihydroergotamine, ergometrine, ergotamine and methylergometrine) are contraindicated with ketoconazole and itraconazole.

The potency of fluconazole as a CYP3A4 inhibitor is much lower and thus its clinical interactions with CYP3A4 substrates are of less magnitude. Doses of less than 200 mg/d are not associated with significant CYP3A4-mediated interactions. So fluconazole ($\leqslant$ 200 mg/d) is a relatively safe alternative azole antifungal when coadministered with statins metabolised by CYP3A4[102]. However, it is a potent inhibitor of CYP2C9. Coadministration of fluconazole with CYP2C9 substrates such as phenytoin, warfarin, fluvastatin and losartan leads to clinically significant drug interactions, whereas concurrent therapy of itraconazole or ketoconazole has minimal effect on PK of CYP2C9 substrates[103,104].

Voriconazole inhibits the activities of CYP2C19, CYP2C9 and CYP3A4. Thus, there is a potential for voriconazole to increase the plasma levels of substances metabolized by these CYPs. Coadministration of voriconazole with CYP3A4 substrates (e.g., terfenadine, astemizole, cisapride, quinidine and sirolimus) is contraindicated. When initiating voriconazole in patients already receiving cyclosporine or tacrolimus, it is recommended that the maintenance dosage of two immunosuppressive agents should be adjusted and that their level be carefully monitored. If patients receiving CYP2C9 substrates (e.g., warfarin, phenytoin or sulphonylureas) are treated simultaneously with voriconazole, pharmacotherapy monitoring and dosage adjustment for these drugs should be implemented accordingly.

DISCUSSION

The relationship between chemical structure and metabolic profile has been described in the above summary on seven classes of drugs for gastrointestinal diseases treatment. The underlying molecular mechanism of interactions between drug and metabolizing enzymes is complex and it determines whether the drug is a substrate or inhibitor of the specific enzyme and how far it influences the enzyme activity.

Comparative molecular field analysis (CoMFA) modelling can reveal the key molecular characteristics of CYP inhibitors. For example, both electrostatic and steric interactions were found to account for the differences in the potencies of drugs to inhibit CYP2B6. The differences in inhibitory effects of H_2-receptor antagonists on CYP enzymes may be attributed to the different ability of substituent to bind to the heme iron in CYP[105]. Cimetidine carries both the imidazole and the cyano groups which strongly bind to the heme iron and are responsible for its prominent interaction potential. In comparison to cimetidine, ranitidine has the following structural characteristics: (1) the imidazole ring is substituted with a furane ring, and (2) the side chain cyano group is substituted with a nitro group. Famotidine, nizatidine and ebrotidine all possess a thiazole nucleus instead of the imidazole ring, and the cyano-group in the side chain is substituted by aminosulfonyl- or nitro group. The affinity of binding with CYP isoenzymes is in the following order: imidazole ring (cimetidine), furane ring (ranitidine), thiazole ring (famotidine, nizatidine and ebrotidine). Roxatidine carries no imidazole group in its chemical structure, so it also has a weak inhibitory effect on CYPs.

The relationship between chemical structure of fluoroquinolone and its interaction magnitude has been determined[106, 107]. Molecular modeling studies showed that it is possible to explain the potency of the quinolones to inhibit CYP1A2 on a molecular level. The keto group, the carboxylate group, and the core nitrogen at position 1 are likely to be the most important groups for binding to the active site of CYP1A2, because of the molecular electrostatic potential in these regions. Fluoroquinolones carrying an alkylated piperazinyl moiety at the position 7 (e.g., ofloxacin, levofloxacin, sparfloxacin, lomexacin and gatifloxacin) or a bulky substituent at the position 8 (e.g., sparfloxacin, lomefloxacin, gatifloxacin and moxifloxacin), are less prone to inhibit CYP1A2 than those without corresponding substituents.

The type of CYP metabolism and degree to which an azole antifungal is metabolized are governed by a number of factors including the physiochemical properties of the drug (lipophilicity) and its PK characteristics. Because ketoconazole and itraconazole are highly lipophilic, their clearance is heavily dependent upon CYP-mediated metabolism[108]. Fluconazole, on the other hand, is relatively less lipophilic and requires less CYP-mediated

metabolism at low dosages (< 200 mg/day). Ketoconazole carries an imidazole ring, whereas itraconazole, fluconazole and voriconazole contain triazole rings. The four azole antifungals are strongly binding to hepatic microsome CYP enzymes in a Type Ⅱ manner (i.e., involving the direct ligation of an azole nitrogen with the iron atom of the haem group in the CYP enzyme), which resulted in the broad-spectrum inhibition of multiple CYP isoforms, although the relative potencies towards the various isoforms vary from drug to drug[109].

Generally, if some type of CYP isoenzyme is the most important determinants of the clearance of a drug, metabolic drug interactions can be anticipated when the drug is coadministered with inducers or inhibitors of this isoenzyme. If this drug has a relative narrow therapeutic window, drug-drug interaction may be of clinical relevance. Morever, obvious inter-individual clinical outcome may be observed in patients if a CYP isoenzyme (the determinant of the clearance of a drug) exhibts polymorphism. Under all these situations in clinical practice, clinicians and pharmcists should show abilities in medication therapy management. Careful observations are needed in using new drugs in view of few clinical experiences.

In conclusions, the metabolic profile includes the fraction of drug metabolized by CYP, CYP reaction phenotype, impact of CYP genotype on interindividual PK variability and CYP-mediated drug-drug interaction potential. Significant differences may be observed with the metabolic profiles of medications for gastrointestinal disease treatment even if they belong to the same therapeutic or structural class. Many events of severe ADRs and treatment failures were closely related to the ignorance of this respect. Clinicians should acquaint themselves with what kind of drug has less interpatient variability in clearance and whether to perform CYP genotyping prior to initiation of therapy. The relevant CYP knowledge also helps clinicians enhance the management of patients on polytherapy regimens, i.e., better anticipate or avoid a drug interaction, choose an alternative agent with lower interaction potential, and perform pharmacotherapy monitoring (e.g., monitoring clinical symptoms and alterations in laboratory values) and dosage adjustment accordingly when concurrent therapy can not be avoided.

CONMENTS

Background

Metabolism by cytochrome P450 (CYP) represents an important clearance mechanism for the majority of drugs, thus affecting their oral bioavailability, duration and intensity of pharmacological action. The metabolic profile of a drug depicts its amount metabolized by CYP, the CYP reaction phenotype, impact of the CYP genotype on interindividual pharmacokinetics variability and CYP-mediated drug-drug interaction potential. It is closely related to the three-dimensional chemical structure of drug and may exhibit significant differences among drugs within the similar therapeutic or structural class, although the efficacy of these similar drugs do not show sharp differences at the dose used clinically. Many events of severe adverse drug reactions and treatment failures are attributed to the ignorance of above issues. In order to promote rational drug use in clinical practice, it is essential to let clinicians know what kind of drug has less interpatient variability in clearance, whether to perform CYP genotyping prior to therapy and how to enhance the management of patients on polytherapy regimens from the perspective of drug metabolism.

Research frontiers

Food and Drug Administration (FDA) published guidance for *in vitro* and *in vivo* drug metabolism/drug interaction studies in the drug development process in 1999. Withdrawals of medications such as terfenadine, astemizole, cisapride, and mibefradil from the market by FDA demonstrate the relevance of metabolic drug-drug interaction profile. Some scientists tried to describe the three-dimensional quantitative structure activity relationships (QSARs) within substrates, inducers and inhibitors of CYP in recent years. There are also sporadic reports on metabolic differences in market products within the similar structural class.

Innovations and breakthroughs

This article is the first systematic summary on metabolic differences in market drug products within the similar therapeutic or structural class for gastrointestinal disease treatment.

Applications

The significance of this article is: (1) it helps doctors realize what kind of drug for gastrointestinal disease treatment has less interpatient variability in clearance and whether to perform CYP genotyping prior to therapy; (2) help doctors enhance management of patients on polytherapy regimens. Doctors will learn to better anticipate or avoid a drug interaction, choose an alternative agent with lower interaction potential, perform pharmacotherapy monitoring and adjust dosage accordingly when concurrent therapy cannot be avoided; and (3) help doctors attach equal importance to medicines for other disease treatment, and finally promote rational drug use in clinical practice.

Terminology

Drug metabolism: the process by which the drug is chemically converted in the body to a metabolite, usually through specialized enzymatic systems. Its rate is an important determinant of the duration and intensity of the pharmacological action of drugs. Cytochrome P450: the most important element of oxidative metabolism of a large number of endogenous compounds (e.g., steroids) and xenobiotics (e.g., drugs). CYP is the standard abbreviation for mammalian cytochrome P450. CYP reaction phenotype: the relative contribution of the CYP isoforms to the metabolic pathways. CYP genotyping: the process of determining the CYP genotype of an individual by molecular biology techniques. It can be used to prospectively identify individuals at risk for adverse drug reactions or therapeutic failure due to altered drug metabolism. $AUC_{po(PM)}/AUC_{po(EM)}$: the ratio of parent drug area-under-the concentration *vs*. time curve after oral dosing (AUC_{po}) derived from poor metabolizers (PM) and extensive metabolizers (EM).

Peer reviews

The review by Zhou *et al* summarizes current literature on seven classes of drugs used in the treatment of gastrointestinal diseases with respect to the clearance of these substances. It is highly interesting and may help physicians to choose an equivalent drug or drug combination in clinical practice.

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CLINICAL RESEARCH

New classification of the anatomic variations of cystic artery during laparoscopic cholecystectomy

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Abstract

AIM: To investigate the anatomic variations in the cystic artery by laparoscopy, and to provide a new classification system for the guidance of laparoscopic surgeons.

METHODS: Six hundred patients treated with laparoscopic cholecystectomy from June 2005 to May 2006 were studied retrospectively. The laparoscope of 30° (Stryker, American) was applied. Anatomic structures of cystic artery and conditions of Calot's triangle under laparoscope were recorded respectively.

RESULTS: Laparoscopy has revealed there are many anatomic variations of the cystic artery that occur frequently. Based on our experience with 600 laparoscopic cholecystectomies, we present a new classification of anatomic variations of the cystic artery, which can be divided into three groups: (1) Calot's triangle type, found in 513 patients (85.5%); (2) outside Calot's triangle, found in 78 patients (13%); (3) compound type, observed in 9 patients (1.5%).

CONCLUSION: Our classification of the anatomic variations of the cystic artery will be useful for decreasing uncontrollable cystic artery hemorrhage, and avoiding extrahepatic bile duct injury.

Key words: Cystic artery; Laparoscopic cholecystectomy; Bile duct injury; Calot's triangle

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INTRODUCTION

Since the advent of laparoscopic cholecystectomy in the last two decades, minimally invasive surgery has evolved through advances in videoscopic technology, instrumentation, and surgical techniques[1-14]. Currently, laparoscopic cholecystectomy is widely accepted as the gold standard in the treatment of cholelithiasis[15-18]. This new technique was initially associated with a significant increase in morbidity, and in particular, in iatrogenic biliary injury and arterial hemorrhage[19-30], perhaps due to a lack of knowledge of the laparoscopic anatomy of the gallbladder pedicle. Therefore, the laparoscopic surgeon has to deal with the new anatomical views and must be aware of the possible arterial and biliary variants.

A good knowledge of Calot's triangle is important for conventional and laparoscopic cholecystectomy. Calot's triangle is an important imaginary referent area for biliary surgery. In 1981, Rocko drew attention to possible variations in the region of Calot's triangle bordered by the cystic duct, common hepatic duct, and lower edge of the liver[31]. In 1992, Hugh suggested Calot's triangle should be renamed the hepatobiliary triangle, with the small cystic artery branches supplying the cystic duct being called Calot's arteries[32].

Cystic artery bleeding is a troublesome complication during laparoscopic cholecystectomy, which increases the rate of conversion to open surgery. If surgery is performed incorrectly, injury to the extrahepatic bile duct or intra-abdominal organs is inevitable. The reported incidence of conversion to open surgery because of blood vessel injuries is approximately 0%-1.9% during laparoscopic cholecystectomy[33], and its mortality is about 0.02%[34]. Safe laparoscopic cholecystectomy demands a good knowledge of the anatomy of the cystic artery and its variations.

The cystic artery has many possible origins, with the right hepatic artery being the most common[35]. The anatomy with respect to the cystic artery between laparoscopic cholecystectomy and open cholecystectomy is different. We investigated the appearance of the cystic artery during laparoscopic cholecystectomy, and proposed a new classification system for the cystic artery during laparoscopic cholecystectomy, according to our practical experience.

MATERIALS AND METHODS

Between June 2005 and May 2006, we undertook a retrospective evaluation of 600 non-emergency patients, 232 men and 368 women, who underwent laparoscopic

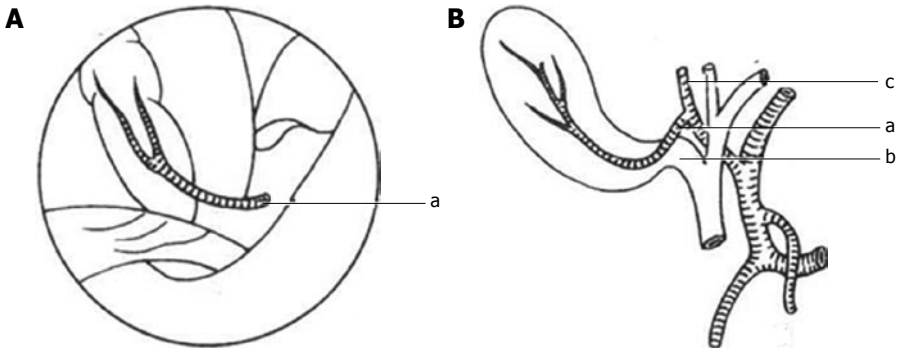


Figure 1 Classical single cystic artery. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct; c: Right hepatic artery.

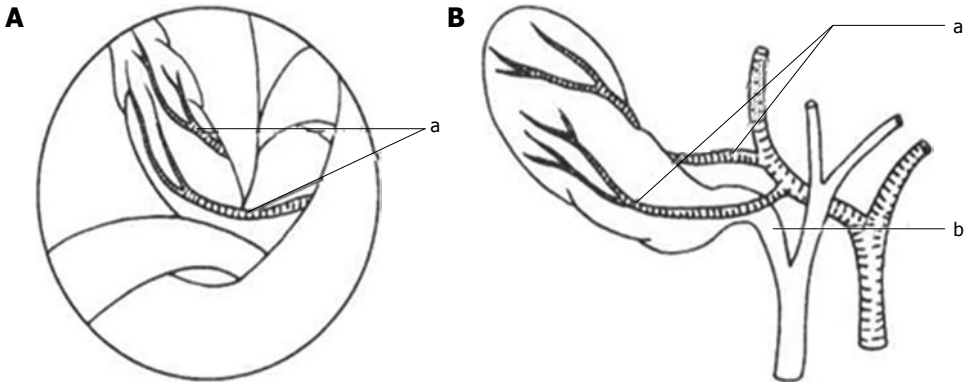


Figure 2 Double cystic artery. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct.

cholecystectomy for different gallbladder diseases, including 530 with cholecystitis and gallstones, and 70 with gallbladder polyps. All of the patients were examined with ultrasound before surgery.

Laparoscopic cholecystectomy was carried out under general anesthesia using the four ports technique. The information of Calot's triangle and distribution of cystic artery on endoscopic visualization was recorded respectively. A laparoscope (Stryker, USA) at 30° tilt angle was used. The anatomical structures were viewed on a three-dimensional video monitor.

RESULTS

Based on our laparoscopic observations, we classified cystic artery anatomy into three groups.

Group I

Group I represents the Calot's triangle type, in which the cystic artery passes through Calot's triangle. The anatomic location of the cystic artery can be found ahead or behind of the cystic duct, and in hepatoduodenal ligament, under laparoscopic observation. This is the most common type and has been reported in about 80%-96% of cases in previous studies[35,36]. We observed this type in 513 of the 600 patients (85.5%). Group I is further subdivided into two subtypes, as follows.

Classical single cystic artery: The cystic artery originates from the right hepatic artery within Calot's triangle. When approaching the gallbladder, the artery is divided into deep and superficial branches at the neck of the gallbladder. The superficial branch proceeds along the left side of the gallbladder. The deep branch runs through the connective tissues between the gallbladder and liver parenchyma. The deep branch gives rise to tiny branches to supply the gallbladder, which anastomose with the superficial branches. This type of cystic artery is laterally positioned from the cystic duct within Calot's triangle during open cholecystectomy, whereas during laparoscopic cholecystectomy it is just behind and slightly deeper than the cystic duct. According to the literature, this type is found in 70%-80% of cases[32,35]. In our study it was recorded in 440 of 600 patients (73.3%) (Figure 1).

Suzuki *et al* has reported a special example of this type. A single cystic artery originates from the right hepatic artery and then hooks around the cystic duct from behind and reappears at the peritoneal surface near the neck of the gallbladder. They named this the cystic artery syndrome[37]. We did not find any examples of this type in our study.

Double cystic artery: The cystic artery also originates from the right hepatic artery, while it divides into the anterior and posterior branches at their cystic artery origin. Congenital absence of the deep branch signifies the existence of another cystic artery, which is occasionally detected by subsequent bleeding control. The posterior cystic artery is very delicate in some cases and is often cut by electrocoagulation during dissection. A double cystic artery has previously been found in 15%-25% of patients[32]. During our laparoscopic cholecystectomy we recorded 73 patients (12.2%) with double cystic artery (Figure 2).

Group II

Cystic artery approaches the gallbladder outside Calot's triangle and cannot be observed within the triangle by

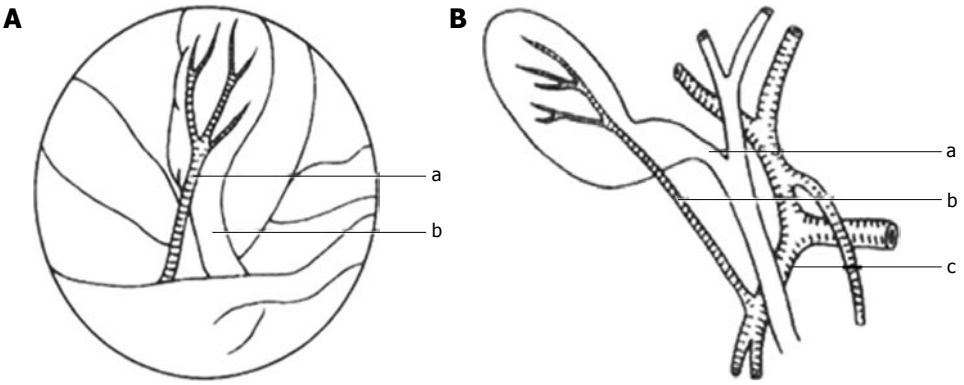
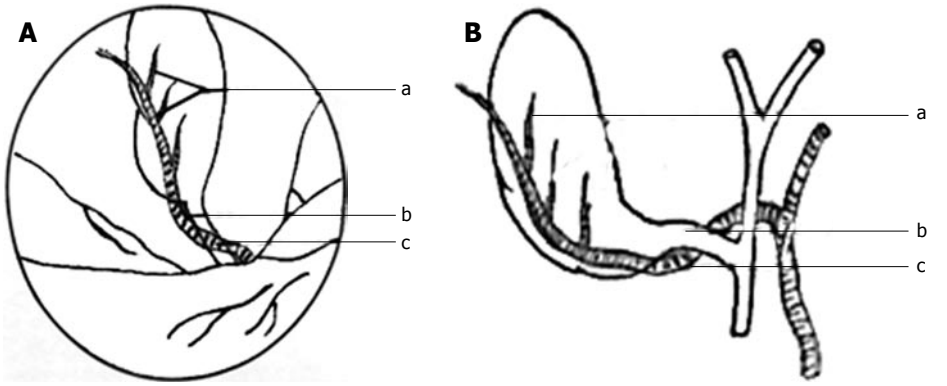


Figure 3 Cystic artery originating from gastroduodenal artery. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct; c: Gastroduodenal artery.



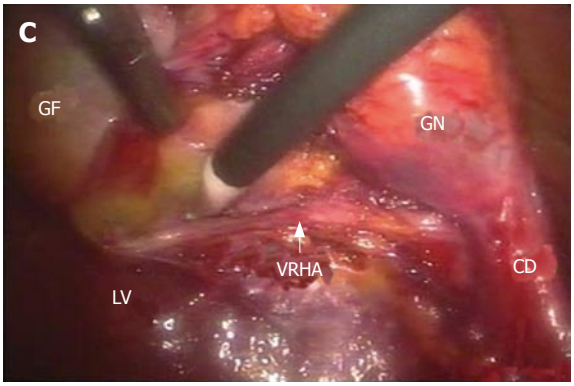


Figure 4 Cystic artery originating from variant right hepatic artery. **A**: Laparoscopic visualization; **B**: Conventional visualization; **C**: Video in operation. a: Cystic artery; b: Cystic duct; c: Variant right hepatic artery; GF: Fundus of gallbladder; CD: Cystic duct; GN: Neck of gallbladder; LV: Liver; VRHA: Variant right hepatic artery.

laparoscopy during dissection. We found 78 patients (13%) in group II during laparoscopic cholecystectomy. This group includes the following four subgroups.

Cystic artery originating from gastroduodenal artery: This type of cystic artery is also called low-lying cystic artery, which does not pass through Calot's triangle but approaches the gallbladder beyond it. In conventional open cholecystectomy it is seen as inferior to the cystic duct, while it usually localizes superficially and anterior to the cystic duct from a laparoscopic viewpoint. Its terminal segment as it approaches the gallbladder is important for laparoscopic surgeons. Because it not only must be manipulated at first, but it is also susceptible to injury and hemorrhage during dissection of the peritoneal folds that connect the hepatoduodenal ligament to Hartman's pouch of the gallbladder or to the cystic duct. This anatomic variation was found in 45 patients (7.5%) in our study (Figure 3).

Cystic artery originating from the variant right hepatic artery: Anatomic variation of the right hepatic artery usually originates from the superior mesenteric artery or aorta[32,38]. It enters Calot's triangle behind the portal vein, and runs parallel to the cystic duct on its passage through the triangle. It can be completely covered by the cystic duct of the gallbladder[32]. We found a very interesting type of right hepatic artery variant. This artery has a long course, approaching the gallbladder deep at its neck, and then passing between the gallbladder and liver parenchyma, extending along the deep right side of the gallbladder, and finally entering the liver parenchyma near the right-lateral side of the gallbladder fundus. It yields multiple small branches to supply the gallbladder at its body, and it is often completely covered by the gallbladder. We should be cautious of this right hepatic artery variation. From the laparoscopic viewpoint it looks like a single large artery. This anatomic variation was found in 18 patients (3%) in our study (Figure 4).

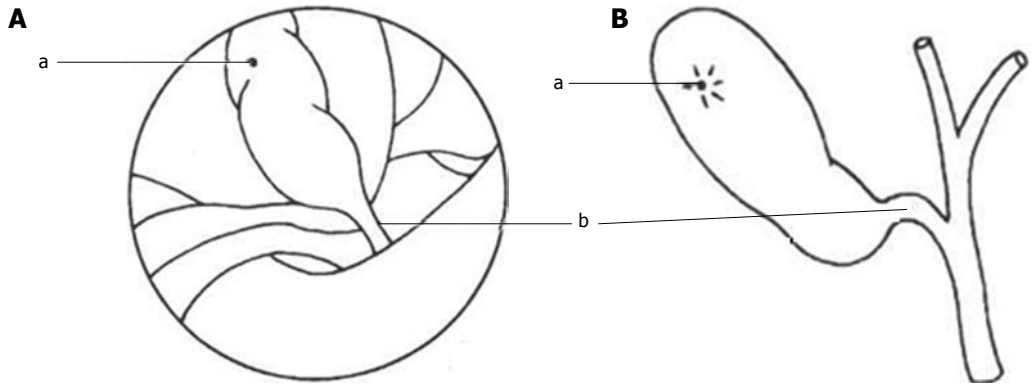


Figure 5 Cystic artery arising from hepatic parenchyma. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct.

Variant right hepatic artery has been shown to have a prevalence of approximate 4%-15%[37,39].

Cystic artery originating directly from the liver parenchyma: This cystic artery pierces the hepatic parenchyma approaching the bladder from the gallbladder bed. It usually situates in the right lateral of the border of gallbladder body and bottom. However, a few are situated in the center of the gallbladder bed or situated left lateral of gallbladder bottom. No other arteries are found within Calot's triangle. This anatomic variation of the cystic artery is not observed until bleeding and is caused by dissection of the gallbladder fundus. It is difficult to explore and requires careful dissection. We found it in 15 patients (2.5%) (Figure 5).

Cystic artery originating from the left hepatic artery: The cystic artery occasionally originates from the left hepatic artery, passes through the liver parenchyma, and reaches the middle of the gallbladder body, at which point it bifurcates into ascending and descending branches. This has a prevalence of 1%[39]. However, we did not find this type of variant cystic artery.

Group Ⅲ

This group has more than one blood supply. We named it the compound cystic artery type. The cystic arteries exist not only in Calot's triangle, but also outside it. We found that nine patients (1.5%) belonged to this group. Five of these patients (0.8%) had a normal single cystic artery in Calot's triangle, and an artery extending along the cystic duct but posterior to it, and some small arteries that passed immediately from the liver parenchyma to the gallbladder. Three of the nine patients (0.5%) had another cystic artery superficial to the cystic duct in addition to the normal cystic artery. Finally, one patient (0.17%) had multiple cystic arteries, including the double cystic artery in Calot's triangle, and one of the arteries crossed anterior to the common bile duct, while another was situated on the right side of the border of the gallbladder body and fundus.

DISCUSSION

Anatomic variations in and around Calot's triangle are frequent (biliary tree, cystic artery)[40,41], and we found them in 20%-50% of patients. Therefore, careful blunt dissection of Calot's triangle is necessary for both conventional and laparoscopic cholecystectomy.

Since the introduction of laparoscopic gallbladder surgery, surgeons have been very interested in gallbladder vascularization. A great number of papers on this issue have been published[36,42-44], although some vagueness still exists, because of the diversity of data and classification. There have been relatively few reports on the laparoscopic anatomy of the hepatobiliary triangle, especially of the cystic artery.

It is important for every laparoscopic surgeon to be familiar with the anatomic variations in the extrahepatic biliary tree and those of the arterial supply of the gallbladder. The possible anatomic position and variations of the cystic artery are difficult to establish before surgery. They were only identified during disconnection of Calot's triangle and the gallbladder. The laparoscopic anatomy of the cystic artery can be considered as a precondition for performing safe laparoscopic procedures. The variations of cystic artery often make surgeons recognize an error, causing them to abscise incorrectly and, subsequently, leading to a hemorrhage. When hemorrhage cannot be controlled, conversion to open cholecystectomy is inevitable.

The position of the cystic artery appears differently during laparoscopic and conventional cholecystectomy for the following reasons. (1) Under laparoscopy, as the gallbladder fundus is pulled, the liver is moved upward, thereby opening the subhepatic space. (2) By pulling Hartman's pouch downward, the anterior aspect of Calot's triangle is presented. On the contrary, by pulling Hartman's pouch upward, the posterior aspect of Calot's triangle is clearly exposed. (3) Better transparency and visualization under laparoscopy facilitates recognition of cystic artery variation by the surgeon.

Previous studies have contained fewer reports on the laparoscopic classification of the cystic artery. Some have divided the cystic artery into low-lying cystic artery and cystic artery originating from variant right hepatic artery. Balija classified cystic artery variations into two groups. Group I comprises five variations of the cystic artery within the hepatobiliary triangle: (a) normal position; (b) frontal cystic artery; (c) backside; (d) multiple; and (e) short cystic artery that arises from an aberrant right hepatic artery. Group Ⅱ consists of variations of the cystic artery that approaches the gallbladder beyond the hepatobiliary

triangle: (a) low-lying; (b) transhepatic; and (c) recurrent cystic artery[36]. Ignjatovic has divided the cystic artery into three types in minimally invasive surgical procedures: type 1 shows normal anatomy; type 2 more than one artery in Calot's triangle; and type 3 no artery in Calot's triangle[45]. However, none of the above classifications satisfies the practical needs of laparoscopic surgery. Based on our experience, the anatomic variations of the cystic artery can be classified into three groups. Group I showed the cystic artery passing within Calot's triangle. It included two types: (1) single cystic artery, found in 440 patients (73.3%); and (2) double cystic artery, observed in 73 patients (12.2%). Group Ⅱ showed the cystic artery situated outside Calot's triangle. This group included four variations: (1) cystic artery originating from the gastroduodenal artery, found in 45 patients (7.5%); (2) cystic artery originating from the variant right hepatic artery, found in 18 patients (3%); (3) cystic artery directly arising from the liver parenchyma, observed in 15 patients (2.5%); and (4) cystic artery originating from the left hepatic artery. Group Ⅲ had a compound appearance, with the variant cystic artery situated not only within Calot's triangle, but also outside it. This classification of cystic artery can help surgeons understand the cystic artery more thoroughly, and may be more practical to use in real operations.

Variations in the cystic artery are miscellaneous, and we must be cautious during the performance of laparoscopic cholecystectomy. Our laparoscopic classification of the cystic artery is very useful for dissection of Calot's triangle, reduces uncontrollable cystic artery hemorrhage, and may be advantageous for avoiding extrahepatic bile duct injury.

COMMENTS

Background

Uncontrolled bleeding from the cystic artery and its branches is a serious problem that may increase the risk of intraoperative injury to vital vascular and biliary structures. Anatomic variations of the cystic artery are frequent and can differ in origin, position and number. We investigated 600 cases treated with laparoscopic cholecystectomy and analyzed the anatomic structure of the cystic arteries under laparoscopic observation. The cystic artery variations were classified into three groups: (1) Calot's triangle, (2) outside Calot's triangle, and (3) compound type. These will be helpful to laparoscopic surgeons.

Research frontiers

Laparoscopic cholecystectomy is widely accepted as the gold standard in the treatment of gallstone disease. However, the incidence of bile duct injury caused by this procedure is more than that caused by conventional cholecystectomy. The main areas of research in laparoscopic cholecystectomy are as follows: (1) biliary injury during laparoscopic cholecystectomy; (2) anatomic characteristics of the cystic artery; (3) intraoperative bleeding during laparoscopic cholecystectomy; (4) cholecystectomy techniques.

Innovations and breakthroughs

There are few reports concerning classification of the cystic artery during laparoscopic cholecystectomy. Some authors have classified it into two groups: Group Ⅰ comprises variations of the cystic artery within the hepatobiliary triangle; and Group Ⅱ comprises variations of the cystic artery that approach the gallbladder beyond the hepatobiliary triangle. Other authors have divided cystic artery variations into three types: type 1, normal anatomy; type 2, more than one artery in Calot's triangle; and type 3, no artery in Calot's triangle. We present a new classification of cystic artery variations based on three groups. Group I shows the cystic artery passing within the Calot's triangle. This includes two types: (1) single cystic artery, and (2) double cystic artery. Group Ⅱ shows the cystic artery situated outside Calot's triangle. This group includes four variations: (1) cystic artery originating from the gastroduodenal artery; (2) cystic artery originating from the variant right hepatic artery; (3) cystic artery arising directly from the liver parenchyma; (4) cystic artery originating from the left hepatic artery. Group Ⅲ shows the cystic artery is compound in nature; the variant cystic artery was situated not only within Calot's triangle, but also outside it. Furthermore, we found a new variant right hepatic artery (Figure 4), which has not been reported before.

Peer review

The authors introduce a new classification of cystic artery variations during laparoscopic cholecystectomy. Their system is different from those reported previously. The new system divides the anatomic variations of the cystic artery into three groups according to the position of the cystic artery relative to Calot's triangle, as seen by laparoscopy. The classification system should be useful to laparoscopic surgeons, and help reduce incidences of bile duct injury and intraoperative bleeding during laparoscopic cholecystectomy.

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RAPID COMMUNICATION

Constitutive androstane receptor agonist, TCPOBOP, attenuates steatohepatitis in the methionine choline-deficient diet-fed mouse

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Abstract

AIM: To ascertain whether constitutive androstane receptor (CAR) activation by 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP) modulates steatohepatitis in the methionine choline-deficient (MCD) diet-fed animal.

METHODS: C57/BL6 wild-type mice were fed the MCD or standard diet for 2 wk and were treated with either the CAR agonist, TCPOBOP, or the CAR inverse agonist, androstanol.

RESULTS: Expression of CYP2B10 and CYP3A11, known CAR target genes, increased 30-fold and 45-fold, respectively, in TCPOBOP-treated mice fed the MCD diet. TCPOBOP treatment reduced hepatic steatosis (44.6 ± 5.4% *vs* 30.4 ± 4.5%, $P < 0.05$) and serum triglyceride levels (48 ± 8 *vs* 20 ± 1 mg/dL, $P < 0.05$) in MCD diet-fed mice as compared with the standard diet-fed mice. This reduction in hepatic steatosis was accompanied by an increase in enzymes involved in fatty acid microsomal ω-oxidation and peroxisomal β-oxidation, namely CYP4A10, LPBE, and 3-ketoacyl-CoA thiolase. The reduction in steatosis was also accompanied by a reduction in liver cell apoptosis and inflammation. In contrast, androstanol was without effect on any of the above parameters.

CONCLUSION: CAR activation stimulates induction of genes involved in fatty acid oxidation, and ameliorates hepatic steatosis, apoptosis and inflammation.

Key words: Apoptosis; CYP4A; Fatty acid oxidation; Inflammation

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INTRODUCTION

Hepatic steatosis or fatty infiltration of the liver has reached epidemic proportions in Western Society. Approximately 30 million adults in the United States have hepatic steatosis, and a subset of these individuals will develop accompanying hepatic inflammation referred to as steatohepatitis[1]. If the steatohepatitis is not associated with significant alcohol intake, it is commonly referred to as nonalcoholic steatohepatitis (NASH). Unfortunately, NASH can progress to cirrhosis and chronic liver failure with considerable morbidity and mortality[2]. Treatment options for NASH are limited and, therefore, there is an unmet need for the pharmacologic treatment of this liver disease.

Although the precise etiopathogenesis of NASH remains to be defined, it is a disease due to perturbations of intermediary fat metabolism. In hepatic steatosis, an excess of non-esterified fatty acids are released from peripheral tissues into the serum[3]. These excess serum-free fatty acids are cleared by the liver where they are esterified and accumulate as neutral fat. The formation of neutral fat is presumably due to a limited capacity to oxidize excess fatty acids. Mechanisms to enhance hepatic fatty acid oxidation are, therefore, a potential strategy to protect the liver from hepatic steatosis. Hepatic fatty acid oxidation occurs by three pathways[4]. β-oxidation is a predominant pathway, which occurs in the mitochondria, and is rate-regulated by carnitine palmitoyltransferase (CPT1) and the mitochondrial trifunctional protein (MPT) complex. Peroxisomal β-oxidation oxidation occurs within peroxisomes and is rate-limited by the peroxisomal L-bifunctional enzyme (L-PBE), acetyl-COA oxidase (ACO), and urate oxidase (UO). The third

pathway is ω-oxidation which occurs in the endoplasmic reticulum. This pathway is dependent upon expression of the cytochrome enzymes CYP4A10 and CYP4A14. Stimulation of these pathways either individually or collectively could help remove excess free fatty acids from the liver and attenuate NASH.

Nuclear receptors are a family of transcription factors, which regulate metabolism of endo- and xenobiotic compounds[5]. In particular, the constitutive androstane receptor (CAR), which is highly expressed in the liver, is a biosensor for endo- and xenobiotic compounds, such as toxic bile acids[6-8] and steroids[9]. CAR mediates the induction of detoxifying enzymes by the widely used antiepileptic drug phenobarbital in humans and by the potent synthetic inducer, 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP) in mice. Once stimulated, CAR increases expression of *CYP2B* genes in the mouse[7,8,10] and human[11]. From a teleological perspective, this nuclear receptor can be viewed as a general hepatoprotective response system, as it detoxifies potentially injurious endo- and xenobiotics. In addition, it serves as a hepatic mitogen and as an anti-apoptotic agent by increasing transcriptional expression of the anti-apoptotic protein, Mcl-1[12,13]. These composite effects eliminate toxins, enlarge the liver[14], and render it resistant to liver injury-all hepatoprotective responses. Whether CAR also protects the liver from injurious endobiotics, such as free fatty acids, is less clear but a viable concept.

Based on the above concepts, we postulated that CAR would protect the liver from the development of steatohepatitis by enhancing processes involved in fatty acid oxidation. Thus, the overall objective of the current study was to ascertain whether CAR activation by TCPOBOP modulates steatohepatitis induced by the methionine choline-deficient diet, a well known murine model of NASH[15]. Two fundamental questions were formulated. In TCPOBOP-treated MCD diet-fed mice: (1) Does CAR activation modify steatosis, and if so, does it alter expression of genes involved in fatty acid oxidation? and (2) does CAR alter apoptosis and inflammation in this model of NASH? The results indicate that CAR activation can stimulate an induction of genes involved in fatty acid oxidation, ameliorating hepatic steatosis, apoptosis and inflammation. These observations suggest CAR stimulation renders the liver resistant to MCD diet-mediated steatohepatitis. An understanding of these mechanisms may allow the development of CAR agonists as therapeutic strategies for NASH.

MATERIALS AND METHODS

Animal models

The care and use of the animals for this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). C57/BL wild-type mice (Jackson laboratories, Bar Harbor, ME), weighing 20-25 g, were fed a methionine choline-deficient (MCD) diet (Harland Tech Lad, Madison, WI) for 2, 3 or 4 wk. This diet rapidly induces steatosis and steatohepatitis in rodents[16]. The mice were maintained in a temperature-controlled, pathogen-free environment and fed a standard rodent chow diet and water ad libitum. To assess the effect of CAR modulation, mice were intraperitoneally (ip) injected with either vehicle (corn oil), 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) (Sigma-Aldrich, St Louis, MO) (3 g/kg) daily for the first 3 d[7] at the starting of the MCD diet, or 5β-androstan-3β-ol (androstanol) (a CAR inverse agonist) (Steraloids, Newport, RI) (100 mg/kg) daily for 3 d at the starting of weeks one and two of the MCD diet (total of 6 injections). Androstanol was used as negative control for CAR target genes. At selected times, the animals were anesthetized with ether, and a hepatectomy was performed prior to euthanasia *via* exsanguination. Blood was drawn *via* the portal vein to examine triglyceride levels. Liver tissue sections were placed in fixative for subsequent microscopic analyses. Liver sections were also subjected to RNA extraction using the Trizol Reagent (Invitrogen, Carlsbad, CA).

Oil red O staining and fat quantification

Liver sections were cut into a thickness of 20 μm in a cryostat, air dried, and then stained with oil red O as per standard techniques[17]. Slides were then viewed under microscopy (Axioplan 2, Carl Zeiss, Inc. Oberkochen, Germany). Digital pictures were captured to quantitate percent fat (red color in area/field area × 100) of digital photomicrographs as described previously[18].

Histology, TUNEL assay and immunohistochemical identification of activated caspases 3/7

Histology, TUNEL assay, and immunohistochemical analysis for activated caspases 3/7 was performed as previously described by us[18]. To accurately quantitate TUNEL-positive and caspase 3/7-positive cells, slides were examined by digital image analysis to quantitate the percent fluorescence/field area of digital photomicrographs as described previously[13].

Measurement of Leukotriene B4

LTB4 was quantitated using a specific ELISA kit (R&D systems) according to the manufacturer's instructions[19].

Immunohistochemistry for CD68

Unstained slides of liver tissue specimens were deparaffinized and hydrated. Antigen retrieval was performed using EDTA (1 mmol/L, pH 8.0). Slides were placed in a vegetable steamer for 40 min at 97℃, followed by a cooling for 2 min. Thereafter, the catalyzed signal amplification system (DAKO, Carpinteria, CA) was used according to manufacturer's instructions. CD68 immunostaining was performed as previously described by us[20].

Real time-polymerase chain reaction (RT-PCR)

Total RNA was obtained from whole liver as previously described by us[21]. After the reverse transcription reaction, the cDNA template was amplified by PCR with Taq polymerase (Invitrogen) and mRNA was quantitated for acetyl-CoA oxidase (ACO), carnitine palmitoyltransferase (CPT1); peroxisomal 3-ketoacyl-CoA thiolase (ketoacyl thiolase), L-peroxisomal bifunctional enzyme (L-PBE), mitochondrial trifunctional protein (MTP) subunits alpha

Table 1 Primer sequences used for RT-PCR

Target	Primer sequence	Product size (bp)
ACO	F 5'-GAACTCCAGATAATTGGCACCTA-3'	75
	R 5'-AGTGGTTTCCAAGCCTCGAA-3'	
CPT-1b	F 5'-ATCATGTATCGCCGCAAACT-3'	85
	R 5'-CCATCTGGTAGGAGCACATGG-3'	
CYP2B10	F 5'-CAA TGGGGA ACG TTG GAA GA-3'	176
	R 5'-TGATGCACTGGAAGAGGA AC-3'	
CYP3A11	F 5'-CTCAATGGTGTGTATATCCCC-3'	423
	R 5'-CCGATGTTCTTAGACACTGCC-3'	
CYP4A10	F 5'-AGTGTCTCTGCTCTAAGCC-3'	180
	R 5'-CCCAAAGAACCAGTGAAA-3'	
Ketoacyl thiolase	F 5'-GCATCCCAGAGACTGTACCTTT-3'	202
	R 5'-GTCTCTGGCCTTCTCGTTCT-3'	
L-PBE	F 5'-TGGCTCTTGGAGGAGGACTAG A-3'	125
	R 5'-AAGCTGCGTTCCTCTTGCA-3'	
MPT-α	F 5'-GGCAGTCTCAGTCGCTTCTC-3'	240
	R 5'-GCACTCCTGATTTGGTCGTT-3'	
MPT-β	F 5'-AGAGCTGCACTTTCGGGTTT-3'	202
	R 5'-CTGTGGTCATGGCTTGGTTT-3'	
PPAR-α	F 5'-GTACGGTGT GTATGAAGCCATCTT-3'	76
	R 5'-GCCGTACGCGATCAGCAT-3'	
Urate oxidase	F 5'-ACCTCCCGTCATTCACTCT-3'	438
	R 5'-ACTGTCCCTGTTATTTTGCC-3'	

ACO: Acetyl-CoA oxidase; CPT1: Carnitine palmitoyltransferase; Ketoacyl thiolase: Peroxisomal 3-ketoacyl-CoA thiolase; L-PBE: L-peroxisomal bifunctional enzyme; MTP: Mitochondrial trifunctional protein subunits alpha and beta; PPAR-α: Peroxisome proliferators-activated receptor-alpha.

and bet, peroxisome proliferators-activated receptor-alpha (PPAR-α) as previously described[22]. Primers used are listed in Table 1. 18S primers (Ambion, Austin TX) were used as a control for RNA isolation and integrity. All PCR products were confirmed by gel electrophoresis. Real-time PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR green as the fluorophore (Molecular probes). The results were expressed as a ratio of product copies per milliliter to copies per milliliter of housekeeping gene 18S from the same RNA (respective cDNA) sample and PCR run.

Statistical analysis

All data represent at least three independent experiments and are expressed as mean ± SE of the mean. Differences between groups were compared using Student *t*-tests and one-way analysis of variance with post hoc Dunnett test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Stimulating CAR target genes in the MCD diet-fed mouse by TCPOBOP

CYP2B10 and CYP3A11 have been identified as CAR target genes[10,23,24]. Therefore, to determine if TCPOBOP activates CAR in the MCD diet-fed mouse, expression of these genes was examined. When TCPOBOP treatment was administered to the MCD diet-fed mice, expressions of both CYP2B10 and CYP3A11 increased (Figure 1A and B). We also observed 45-fold and 30-fold elevations in CYP2B10 mRNA level in TCPOBOP-treated standard

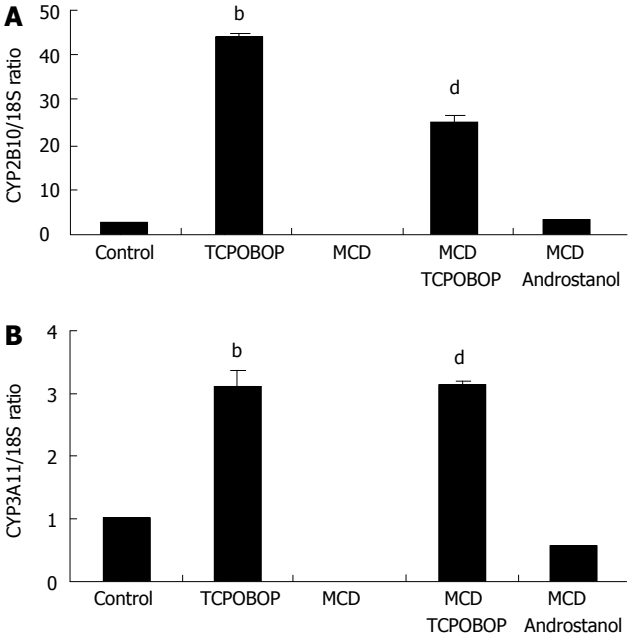

Figure 1 Over-expression of CAR target genes in MCD diet-fed mice treated with TCPOBOP. CAR activation was assessed by measuring CYP2B10 and CYP3A11 (CAR target genes) expression in whole liver from vehicle-treated and TCPOBOP-treated chow-fed and MCD diet-fed mice. Expression was measured by real-time PCR and normalized as a ratio using 18S mRNA as housekeeping genes. A value of 1 for this ratio was arbitrarily assigned to the data obtained from vehicle-treated $CAR^{+/+}$ mice. (**A**) CYP2B10 and (**B**) CYP3A11 mRNA expressions were increased in TCPOBOP-treated (3 mg/kg ip for 3 d) chow-fed mice ($^bP < 0.01$ for CYP2B10 and CYP3A11 compared to control) and MCD diet-fed mice ($^dP < 0.01$ for CYP2B10 and CYP3A11 compared to untreated). This phenomenon was abated by treatment with a CAR inhibitor androstanol (100 mg/kg ip) daily for 3 d at the starting of weeks one and two of the MCD diet (total of 6 injections), ($n = 5$ in each group).

diet-fed mice and MCD diet-fed mice, respectively. CYP3A11 mRNA levels were also similarly increased in TCPOBOP-treated animals. Administration of the CAR inverse agonist, androstanol[10,25], did not alter expression of these gene products (Figure 1A and B). Androstanol was, therefore, used as a negative control for CAR target genes in this model, as it blocks basal activity of CAR[26]. Unexpectedly, CYP2B10 and CYP3A11 mRNA levels remained elevated for 2 wk after initial TCPOBOP administration, mRNA expression of these target enzymes began to decrease by week three (down to 7-fold elevation) and further decreased by week four (down to 6-fold elevation) (data not shown). This data illustrates that TCPOBOP is a potent CAR agonist in the fatty liver and its effects are long-lasting after initial treatment.

Effects of TCPOBOP on hepatic and serum lipid content in MCD diet-fed mice

Animals treated with TCPOBOP and fed the MCD diet for 2 wk had 25% less hepatic steatosis than animals fed the MCD diet alone (Figure 2A). Interestingly, MCD diet-fed animals treated with androstanol had 15% more hepatic steatosis per surface area than untreated MCD diet-fed littermates. Of note, the fat pattern observed in the androstanol-MCD group was homogenously macrovesicular, where in untreated MCD diet-fed animals, the steatosis pattern was heterogeneous with both

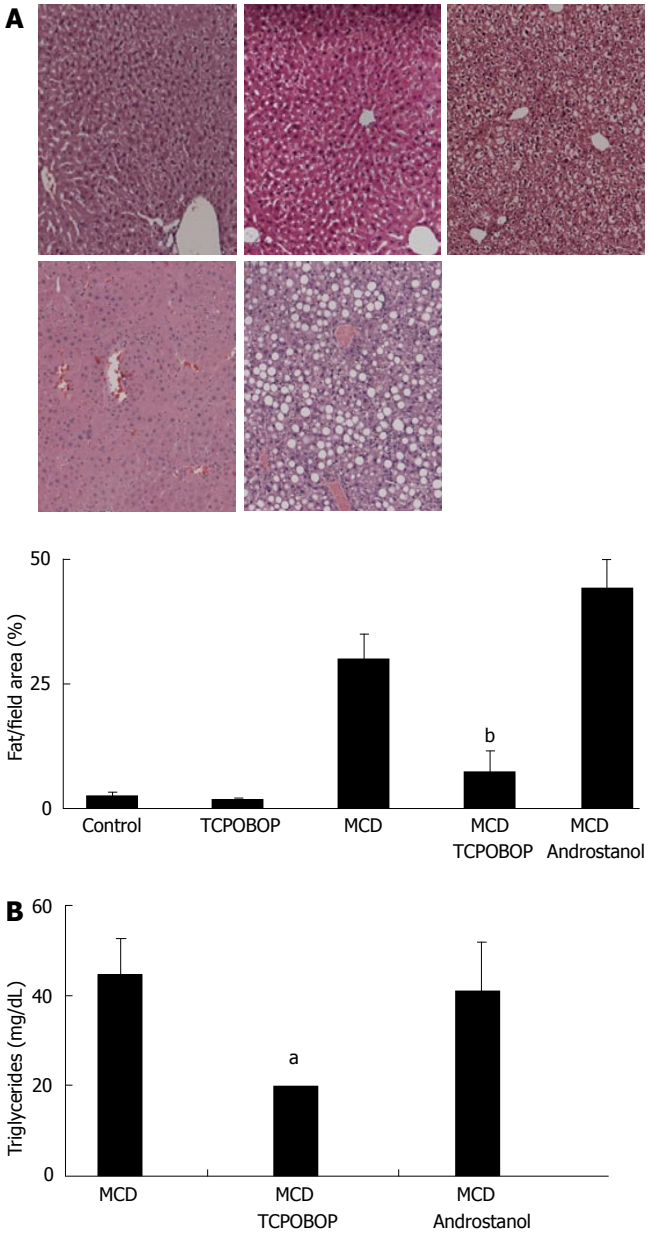

Figure 2 Hepatic and serum fat content reduced by TCPOBOP treatment in the MCD diet-fed mice. (**A**) Top panel, from left to right: Fixed liver specimens from vehicle-treated chow-fed mice, TCPOBOP-treated (3 mg/kg ip for 3 d) chow-fed mice, vehicle-treated (corn oil) MCD diet-fed diet mice for 2 wk, TCPOBOP-treated MCD diet-fed mice for 2 wk; CAR inhibitor, androstanol-treated MCD diet-fed mice for 2 wk were stained by conventional H&E. Lower panel: Quantitation of hepatic fat content by Oil red O staining. Note the marked reduction of hepatic fat in the TCPOBOP-treated MCD diet-fed mice (^{b}P = 0.001); (**B**) Serum triglyceride levels from mice fed the MCD diet for 2 wk with either TCPOBOP or androstanol treatment. Treatment with TCPOBOP reduced serum triglyceride levels by half (^{a}P = 0.03).

microvesicular and macrovesicular steatosis. Administration of TCPOBOP to the MCD diet-fed animals also markedly lowered serum triglyceride levels by 2-fold (Figure 2B). As expected, treatment with androstanol did not alter serum triglyceride levels. Collectively, these data demonstrate that TCPOBOP treatment decreased hepatic fat accumulation and improved serum triglyceride levels.

Modulation of hepatic expression of genes involved in fatty acid oxidation by TCPOBOP

The reduction in hepatic steatosis was consistent with stimulation of fatty acid oxidation by TCPOBOP. Therefore, we evaluated the effect of TCPOBOP on expression of several target genes involved in fatty acid oxidation (Table 2). In the liver, microsomal, mitochondrial and peroxisomal fatty acid oxidation systems are regulated by PPAR-α. PPAR-α mRNA expression levels were not altered by treatment with TCPOBOP in the MCD diet-fed animal (data not shown). However, CYP4A10 and CYP4A14, target enzymes for PPAR-α involved in the microsomal ω-oxidation system, were altered by TCPOBOP administration (Table 2). CYP4a10 mRNA expression was increased 4-fold ($P < 0.001$), while, CYP4A14 mRNA expression was actually decreased by TCPOBOP treatment ($P < 0.01$). L-PBE and peroxisomal 3-ketoacyl-CoA thiolase (ketoacyl thiolase), genes involved in peroxisomal β-oxidation were also significantly modified by TCPOBOP. mRNA expression was increased 6-fold for L-PBE, ($P < 0.001$) and 2-fold for ketoacyl thiolase ($P < 0.05$), when compared to MCD diet-fed untreated animals (Table 2). Two key genes involved in mitochondrial β-oxidation, carnitine palmitoyltransferase (CPT1) and mitochondrial trifunctional protein (MTP), showed no significant difference between TCPOBOP-treated and -untreated MCD diet-fed mice. These data demonstrated a PPAR-α-like effect on microsomal ω-oxidation and peroxisomal β-oxidation by TCPOBOP, which may confer protection against steatosis in the MCD diet-fed animal.

Table 2 Effect of TCPOBOP on genes involved in fatty acid oxidation

Type of fatty acid oxidation	Gene	MCD	MCD-TCPOBOP[a]	Fold-change	*P* value
ω-oxidation	CYP4A10	1.40 ± 1.69	6.07 ± 1.73	4.3	0.001
(ER)	CYP4A14	0.81 ± 0.04	0.07 ± 0.02	-11.6	0.008
β-oxidation	L-PBE	0.07 ± 0.01	0.39 ± 0.01	5.6	0.0004
(peroxisome)	Ketoacyl thiolase	0.12 ± 0.03	0.28 ± 0.06	2.3	0.04
	ACO	0.25 ± 0.14	0.32 ± 0.08	1.3	NS
	Urate oxidase	0.29 ± 0.02	0.16 ± 0.02	-1.8	NS
β-oxidation	CPT1	0.79 ± 0.21	0.77 ± 0.19	-1.0	NS
(mitochondria)	MTP-α	0.60 ± 0.30	0.88 ± 0.71	1.5	NS
	MTP-β	0.50 ± 0.40	0.59 ± 0.07	1.2	NS

Values are expressed as mRNA expression/18S ratio ± SD. [a]Animals fed methionine choline-deficient (MCD) diet for 2 wk and pre-treated with the CAR agonist, TCPOBOP (3 mg/kg ip for 3 d at starting of diet). NS: Not significant; ER: Endoplasmic reticulum; L-PBE: L-peroxisomal bifunctional enzyme; ACO; Acetyl-CoA oxidase; Ketoacyl thiolase: Peroxisomal 3-ketoacyl-CoA thiolase; CPT1: Carnitine palmitoyltransferase; MTP: Mitochondrial trifunctional protein subunits alpha and beta.

Alteration of apoptosis and inflammation in the MCD diet-fed animals by TCPOBOP treatment

Although TCPOBOP reduced hepatic steatosis, this reduction may or may not be sufficient to attenuate liver inflammation. Therefore, we assessed the effect of TCPOBOP on hepatocyte apoptosis and its effect on inflammation by quantitating macrophage/Kupffer cell numbers. Apoptosis was assessed by quantitating TUNEL- and caspase 3/7-positive cells. TUNEL- and caspase 3/7-positive cells were reduced in TCPOBOP-treated MCD diet-fed mice; this reduction of apoptosis was not observed with androstanol treatment (Figure 3A and B). Hepatic inflammation was assessed by

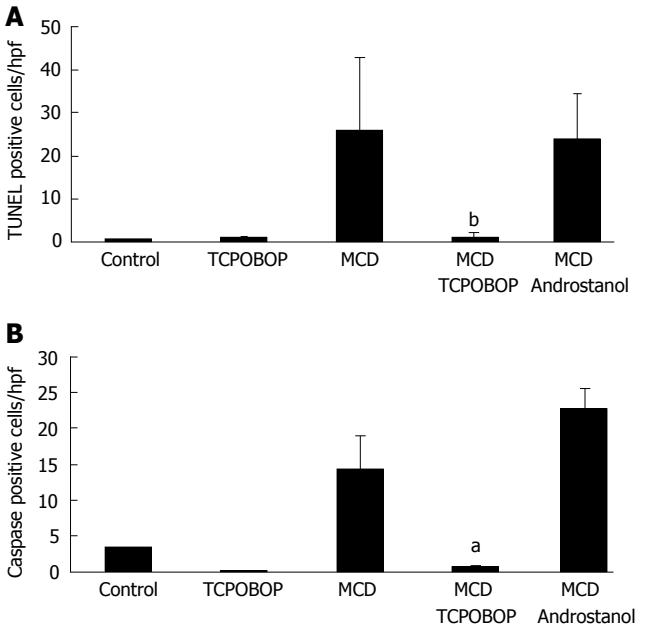

Figure 3 Hepatocyte apoptosis is attenuated by TCPOBOP in the MCD-diet fed animal. (**A** and **B**) Fixed liver specimens were analyzed by TUNEL and immunofluorescence for active caspase 3/7 to identify apoptotic liver cells. The percent/field area of TUNEL- (^bP = 0.01) and active caspase 3/7-positive cells (^aP = 0.03) were significantly higher in vehicle-treated MCD diet-fed mice than TCPOBOP-treated (3 mg/kg ip for 3d) MCD diet-fed mice (n = 5 in each group).

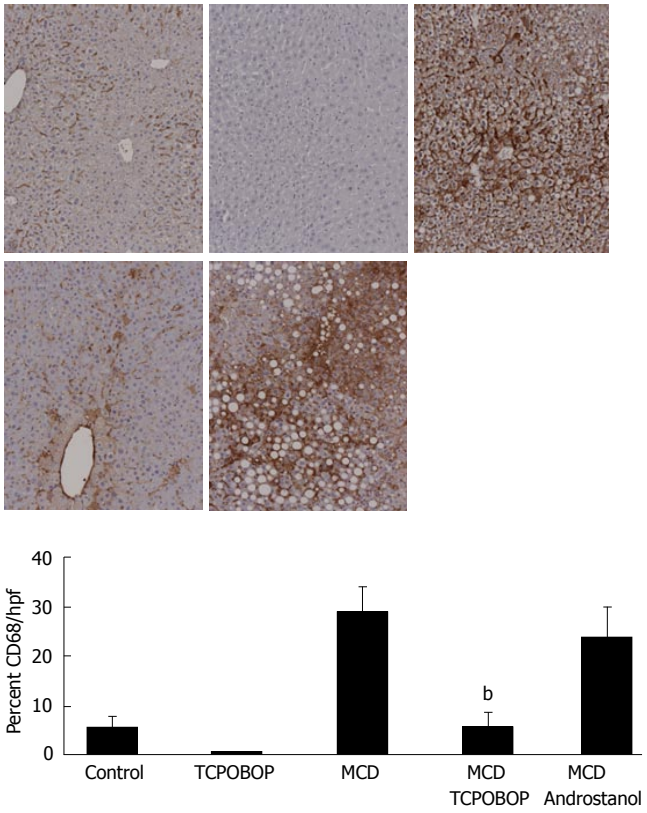

Figure 4 Hepatic inflammation attenuated by TCPOBOP treatment in the MCD diet-fed mice. Top panel: Representative photomicrographs of immunohistochemistry for CD68, a marker of Kupffer cells; Lower panel: The percentage of CD68-positive areas in the liver sections was quantitated using digital image analysis. Immunoreactivity for CD68 was significantly reduced in TCPOBOP-treated MCD diet-fed mice as compared with vehicle- and androstanol-treated mice (^bP = 0.002) (n = 5 in each group).

immunohistochemical staining for CD68, a marker for macrophage/Kupffer cells[27] (Figure 4). CD68-positive cells were less abundant in the MCD diet-fed mice treated with TCPOBOP as compared with the untreated mice on MCD diet; androstanol treatment did not reduce the number of CD68 immunoreactive cells. Consistent with these findings, leukotriene B4 (LTB4), a potent chemotactic agent that initiates, sustains and amplifies the inflammatory response, was also reduced in TCPOBOP-treated MCD diet-fed animals (55.6 ± 2.8 pg/mL) as compared to untreated MCD diet-fed mice (266.9 ± 12.1 pg/mL) (P = 0.001). These results suggest that TCPOBOP pre-treatment abrogates apoptosis and inflammation induced by the MCD diet.

DISCUSSION

The principle findings of this study relate to the effect of CAR modulation of steatohepatitis. The observations suggest TCPOBOP stimulation of CAR in the MCD diet-fed mice model of NASH: (1) reduces hepatic steatosis; (2) increases expression of genes involved in microsomal ω-oxidation and peroxisomal β-oxidation pathways; (3) and reduces hepatic inflammation.

We utilized the MCD animal model of steatosis for this study. The MCD diet stimulates steatosis by inhibition of fatty acid oxidation[15]. This animal model of hepatic steatosis is unique in that mice fed the MCD diet develop inflammation, thereby mimicking human NASH. Recently, peroxisome proliferator-activated receptor α (PPAR-α) agonists have been shown to be useful in ameliorating steatohepatitis induced by the MCD diet in mice by increasing genes involved in fatty acid oxidation[28,29]. PPAR-α is a transcription factor belonging to the nuclear receptor superfamily (NRSF) that increases hepatic uptake and breakdown of fatty acids by up-regulating genes involved in peroxisomal and mitochondrial β-oxidation and microsomal ω-oxidation[5]. In our current study, TCPOBOP administration demonstrated a PPAR-α-like effect on fatty acid microsomal ω-oxidation and peroxisomal β-oxidation enzymes, by increasing expression of CYP4A10, L-PBE and 3-ketoacyl-CoA thiolase. Our observations are consistent with findings from other studies in which protection from hepatic steatosis was afforded by changes in fatty acid microsomal ω-oxidation and peroxisomal β-oxidation without alteration of genes involved in the mitochondrial β-oxidation pathway[30]. These results indicate that upregulation of peroxisomal and microsomal enzymes was primarily responsible for the CAR-dependent reduction in hepatic steatosis.

Our data indicated that CAR activation by TCPOBOP had a PPAR-α-like effect on enhancing expression of genes involved in fatty acid oxidation. How CAR activation mimics PPAR-α activation is unclear, but suggests a potential interaction between the two nuclear receptors. CAR binds to its cognate DNA motif as a heterodimer with the retinoid X receptor (RXR), which serves as a common heterodimerization partner for other nuclear receptors in the superfamily, such as PPAR-α, pregnane X receptor (PXR), liver X receptor (LXR), and farnesol

X receptor (FXR)[31]. Although evidence exists for cross-talk between nuclear receptors of this superfamily[32,33], it is unknown if there is a direct link between CAR and PPAR-α activation. CAR could potentially bind to PPAR-α/RXR heterodimer, as LXR has been shown to bind directly to the PPAR-α/RXR complex[34]. This may afford CAR's ability to enhance expression of PPAR-α target genes involved in fatty acid oxidation. CAR has been shown to increase expression of gene targets otherwise thought to be regulated by other nuclear receptors. Recent experiments have illustrated CAR's ability to activate CYP3A, normally thought as the target of PXR[35]. Further studies are necessary to define the nature of the potential cross-talk between CAR and PPAR-α.

Apoptosis of markedly steatotic hepatocytes may incite the inflammatory response in NASH[36]. Apoptotic markers were reduced in TCPOBOP-treated MCD diet-fed animals in the present study. Indeed, previous work by us[13] has demonstrated CAR to have anti-apoptotic properties. In these studies, CAR activation by TCPOBOP depleted hepatocytes of the pro-apoptotic proteins Bak and Bax and increased expression of the potent anti-apoptotic protein Mcl-1 by directly promoting Mcl-1 transcription. These CAR-dependent processes rendered the liver resistant to death receptor-induced liver injury by Fas. The steatotic liver is highly susceptible to Fas-mediated apoptosis[37]. Perhaps, the CAR-induced resistance to Fas or other death ligands, such as tumor necrosis factor-alpha (TNF-α), accounts for its cytoprotective effects on the MCD diet-fed mouse. Finally, we noted that CAR can modify bile acid metabolism, and the role of bile acids in lipotoxicity remains unexplored[38].

The accumulation of fat in hepatocytes (steatosis) and the onset of steatohepatitis may reflect successive stages in fatty liver disease. The "two-hit" hypothesis postulates that the steatotic liver is susceptible to secondary insults including vulnerability to reactive oxygen species, tumor necrosis factor-α and other cytokines, which ultimately culminates in a sustained inflammatory response[39]. TCPOBOP treatment of MCD diet-fed mice resulted in a marked reduction in steatosis as well as inflammation. CAR-stimulated MCD diet-fed animals had decreased hepatic macrophage/Kupffer cell numbers and less circulating serum leukotriene B4 (LTB4). LTB4 is a potent chemotactic agent that initiates, sustains and amplifies the inflammatory response. Catabolism of LTB4 occurs in hepatocytes, as the liver is the principle organ for clearance of LTB4 from circulating blood[40]. In the liver, degradation of the fatty acid-like derivative LTB4 is modulated by PPAR-α regulation of microsomal ω-oxidation and peroxisomal β-oxidation pathways. Upregulation of PPAR-α-regulated genes involved in fatty acid oxidation by CAR activation may have attributed to a reduction in the inflammatory response, in part by enhancing LTB4 hepatic clearance.

In summary, our findings suggest that CAR activation is sufficient to attenuate hepatic steatosis, apoptosis and inflammation in the MCD diet-fed mice. The CAR-dependent PPAR-α-like stimulation of genes involved in microsomal ω-oxidation and peroxisomal β-oxidation pathways may, in part, be responsible for the hepatic cytoprotective effects observed in this model. These data also implicate a mechanistic link between fatty acid oxidation, hepatocyte apoptosis and Kupffer cell infiltration or inflammation. These preclinical studies suggest the employment of CAR agonists in the treatment of fatty liver diseases merits further attention.

ACKNOWLEDGMENTS

We appreciate the excellent secretarial service provided by Erin Bungum.

COMMENTS

Background

Nonalcoholic steatohepatitis (NASH) is a progressive spectrum of fatty liver disease for which there are limited therapeutic options. Apoptosis or programmed cellular death is a prominent histopathologic feature of NASH and correlates with disease severity. Recently, the constitutive androstane receptor (CAR), a nuclear receptor, has been reported to promote hepatic cytoprotection against apoptosis. Therefore, we hypothesized that CAR may ameliorate apoptosis induced by NASH.

Research frontiers

The incidence of nonalcoholic steatohepatitis is increasing with the rise in obesity over the last decade. New therapeutic regimens to slow down the process of fibrosis and cirrhosis in these patients will be important.

Innovations and breakthroughs

There is a prominent role for CAR cytoprotection against Fas-mediated hepatocyte injury *via* a mechanism involving upregulation of Mcl-1 (anti-apoptotic proteins) and, likely, downregulation of Bax and Bak (pro-apoptotic proteins).

Applications

At this time the use of CAR agonists to reduce the liver injury caused by NASH-induced hepatocyte apoptosis is still in its early phase of development. We need more data to fully evaluate its role in NASH.

Terminology

CAR is highly expressed in the liver and the small intestine, two key tissues expressing xenobiotic metabolizing enzymes, and mediates the induction of their expression by the widely used antiepileptic drug, phenobarbital (PB) and the potent synthetic inducer 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP). TCPOBOP is an agonist ligand for CAR. PB induces its nuclear translocation, which results in increased expression of CAR target genes. The nuclear receptor, peroxisome proliferator-activated receptor alpha (PPAR-α), mediates many, if not all, of the adaptive consequences of peroxisome proliferator exposure in the liver, including alteration in lipid metabolism genes, hepatomegaly, and increases in liver tumors. Apoptosis is executed by caspases, a family of proteases that sequentially disassemble a cell. The pathways leading to caspase activation are dependent on the cytotoxic stimulus. Cytotoxic stress activates caspases by initiating signaling pathways that converge on the Bcl-2 family of proteins. After apoptotic stimulation, changes in the balance between pro- and anti-apoptotic members of this family lead to alteration of mitochondrial pore structure or integrity and permeabilization and release of proteins that promote cell death.

Peer review

This paper describes a series of experiments investigating the modulation of the CAR of steatotohepatitis in experimental rat model of NASH. The paper is well written and the methodology seems adequate. There are some issues that have to be addressed.

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RAPID COMMUNICATION

Viral blips during long-term treatment with standard or double dose lamivudine in HBe antigen negative chronic hepatitis B

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Abstract

AIM: To evaluate safety and effect on hepatitis B virus (HBV) suppression of a long-term treatment with lamivudine (LAM) at standard (100 mg/d) or double (200 mg/d) dose in chronic hepatitis B.

METHODS: This was a case study with matched controls (1:3) in patients with chronic hepatitis B with anti-HBe antibodies.

RESULTS: Twelve patients received LAM 200 mg/d and 35 LAM 100 mg/d, for a median of 28 mo. A primary response (PR; i.e., negative HBV-DNA with Amplicor assay) was achieved in 100% of LAM-200 patients and 83% of LAM-100 patients. A virological breakthrough occurred in 16.7 and 24.7%, respectively, of the PR-patients, with the appearance of typical LAM resistance mutations in all but one patient. Viremia blips (i.e., transient HBV-DNA below 80 IU/mL in patients who tested negative at Amplicor assay) were detected using a real time polymerase chain reaction (PCR) and occurred in seven out of nine patients with subsequent BT and in four out of 32 patients with end-of-study response (77.7% *vs* 12.5%; $P = 0.001$) at chi-square test). At the end of the study, 51.4% of LAM-100 patients and 83.3% of LAM-200 patients had remained stably HBV-DNA negative. Double-dose LAM was well tolerated.

CONCLUSION: Long-term treatment of anti-HBe positive chronic hepatitis B with double dose lamivudine causes a more profound and stable viral suppression as compared to conventional treatment.

Key words: Lamivudine; Hepatitis B; Chronic; Hepatitis; Polymerase chain reaction

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INTRODUCTION

Lamivudine (LAM) treatment has changed the therapeutic approach to chronic hepatitis B (CHB), since it has a potent antiviral effect and an excellent tolerability profile[1-4]. In chronic hepatitis B with anti-HBe antibodies, lamivudine must be administered indefinitely, since drug discontinuation causes an immediate rebound in the vast majority of patients[5,6]. Unfortunately, the drug has a low genetic barrier which causes the emergence of resistant mutants and disease resurgence at a rate of approximately 15% a year[7-10]. In addition, a percentage of patients ranging from 11% to 32% are primary non-responders to treatment[11-14], which limits further the efficacy of lamivudine in this setting.

At present, about 80% of patients with CHB in the Mediterranean area lack HBeAg in serum and present anti-HBe antibodies with the presence of HBV-DNA in serum[15]. This percentage is expanding in other parts of the world[16], due to the control measures against HBV and the subsequent decline in the proportion of hepatitis B virus (HBV) infections in young patients.

Lamivudine is registered for the treatment of chronic hepatitis B at a standard daily dose of 100 mg. This dosage derives from studies which compared the efficacy of 25, 100 and 300 mg/d given for 12-24 wk and found that viral suppression was similar at 100 and 300 mg[17,18]. As a consequence, 100 mg was adopted as the standard of therapy and the potential of higher doses in improving long-term outcomes has never been investigated.

In this pilot study we investigated the feasibility and the effects on viral suppression of a long-term treatment with a daily dose of 200 mg lamivudine in patients with HBeAg-negative chronic hepatitis B compared with matched patients treated with the standard dose.

MATERIALS AND METHODS

The study enrolled consecutive patients with HBeAg-negative, anti-HBe positive CHB between June 1999 and December 2002. The patients had to be negative for anti-HDV, anti-HCV and anti-HIV antibodies and present HBV-DNA in serum at levels $> 2 \times 10^4$ IU/mL. Basal evaluation comprised routine liver function tests, abdominal ultrasound (US) and liver biopsy. Histological specimens were evaluated using the Ishak's scores for necroinflammation and fibrosis[19]; minimal requirements for a specimen to be evaluated were a length of at least 2 cm and the presence of $\geqslant$ 10 portal tracts.

Twelve consecutive patients received lamivudine at the daily dose of 200 mg (LAM 200; 100 mg b.i.d.). As a reference group, we selected three age matched patients for each case, with anti-HBe positive CHB treated in the same period with the standard dose of 100 mg according to an open label, long-term study. In both cases and controls, the therapy was continued indefinitely; the therapy was stopped in patients who did not clear HBV-DNA after 12 mo of treatment. The study was closed on June 30, 2005.

The patients were followed-up monthly, including medical examination and alanine aminotransaminase (ALT) determination; at each visit a serum sample was stored at -40℃ for HBV-DNA testing. An abdominal US was performed every six months. Lamivudine tablets were dispensed directly monthly.

HBV-DNA analysis

Routine quantitative testing for HBV-DNA in serum was performed using Amplicor HBV Monitor (Roche, Branchburg, NJ, USA; detection limit 80 IU/mL).

In addition, analysis of HBV-DNA in serum was performed by the use of a Real Time PCR (RT-PCR) and Sybr Green as a fluorescent intercalating agent of the double strand DNA. The reference standards used for the external calibration were the WHO hepatitis B virus DNA International Standards No. 97/746 and the working reagent HBV No. 98/780, both provided by the National Institute of Biological Standards and Controls. HBV-DNA was extracted from serum by a slightly modified guanidinium thiocyanate method. Briefly, 100 μL of serum or plasma were added to 400 μL of the extraction solution and 0.5 mL of 2-propanol were added to the tube. The mixture was centrifuged for 10 min, then supernatant was removed and pellets washed twice with 70% ethanol. Each sample was dissolved in 50 μL sterile water and 10 μL were used for PCR (sensitivity = 10 IU/mL).

Processed specimens were added to 40 μL of an amplification buffered (pH = 8.0) mixture containing 0.5 μmol/L each of the reverse and forward primers directed toward the highly conserved HBV pre-core/core region and Sybr Green (BMA Molecular Probe), 2.5 IU of Taq Gold (Applied Biosystem) and 0.5 IU of Uracil DNA Glycosylase (Amersham Life Science - USB). The PCR process was carried out with the following amplification cycles: 50℃ for 2 min; 94℃ for 10 min; two cycles, each of 1 min duration, at 94℃, 60℃ and 72℃; 38 cycles at 94℃, 60℃ and 72℃, of 20 s each. Real time detection was performed using a ABI PRISM 7000 Sequence Detection System (Applied Biosystem).

HBV polymerase mutant and HBV genotype assay

Polymerase mutants conferring LAM resistance were detected using a commercial assay (InnoLipa HBV-DR, Innogenetics, Gent, Belgium) in all patients who presented serum HBV-DNA reappearance while on therapy and in those who remained viremic from the beginning of the therapy. HBV genotypes were identified using the INNOLIPA HBV-Genotyping test (Innogenetics, Gent, Belgium).

Therapy outcome definitions

Virological response (VR) to treatment was a fall in HBV-DNA below 80 IU/mL with or without a biochemical response (BR). Patients who remained constantly serum HBV-DNA positive over a 12 mo therapy period were defined as primary non-responders (PNR). A virological breakthrough (VBT) was defined as an increase $\geqslant$ 1 log in serum HBV-DNA in a previous responder patient while continuing LAM therapy; a biochemical breakthrough (BBT) was an ALT elevation above 2 × in a virological BT. An ALT flare was an ALT elevation > 500 U/L. A viral blip was a transient HBV-DNA below 80 IU/mL detected by RT-PCR in patients who tested negative at Amplicor assay. Complete viral suppression was a stably undetectable HBV-DNA in serum using RT-PCR.

Safety

A clinical examination was performed monthly. At the same time, differential blood count and serum biochemistry were performed, including serum amylase, lipase and creatinine phosphokinase (CPK) determination. Any adverse event was registered.

Statistical analysis

The data were analyzed according to an intention to treat procedure. Patients who were lost at follow-up were considered as non-responders. Chi-square and Fisher's exact test were used to analyze categorical variables and Student's *t* test for continuous data. The data were analyzed using SPSS software, version 12. The study was approved by the local Ethics Committee.

RESULTS

Twelve patients received LAM 200 mg/d and 35 patients LAM 100 mg/d (one patient receiving 200 mg LAM was matched with two controls only). The basal characteristics of the patients are reported in Table 1. There was no difference between the groups relating to demographic data and ALT values, serum HBV-DNA values and liver histology. Overall, a liver biopsy taken within 18

Table 1 Baseline characteristics of the patients

	Lam-200	Lam-100	*P*
Number of patients	12	35	NS
Age (median; range)	44.5 (32-60)	44 (23-72)	NS
BMI median (range)	24.3 (23.2-28.7)	25.5 (20.9-34.3)	NS
Gender (M/F)	11/1	26/9	NS
Histology			NS
Minimal-Mild	1 (14%)	6 (22%)	
Moderate-Severe	3 (43%)	7 (26%)	
Cirrhosis	3 (43%)	14 (52%)	
ALT (x n.v.)			NS
< 3	4 (33.3%)	16 (50%)	
3-5	3 (25%)	5 (15.6%)	
> 5	5 (41.7%)	11 (34.4%)	
HBV-DNA IU/mL median (range)	1.06×10^6 (3.2×10^4-3.8×10^6)	6×10^5 (2.4×10^4-3.5×10^6)	NS
Months of therapy median (range)	28 (8-50)	28 (9-48)	NS

Table 2 Virological responses in the two treatment groups *n* (%)

	Lam-100 (*n* = 35)	Lam-200 (*n* = 12)
Primary responses	29 (82)	12 (100)
Breakthroughs	7 (24.6)	2 (16.6)
End of study responses (amplicor)	22 (62.8)	10 (83.3)
Stably undetectable HBV-DNA by RT-PCR	18 (51.4)[a]	10 (83.3)[a]

[a]*P* = 0.051; 51.4% *vs* 83.3%.

mo before therapy was available in 34 patients; 50% of whom had cirrhosis. In both groups median treatment duration was 28 mo. Based on Roche Monitor assay, a primary VR was observed in all the patients treated with 200 mg lamivudine and in 83% of those treated with the standard regimen (Table 2). In both groups, median HBV-DNA disappearance time was 3 mo and in no patients did HBV-DNA become undetectable at Monitor test later than the sixth months of therapy. Primary non-response was observed in six patients, all belonging to the 100 mg group. In PNRs LAM therapy was discontinued after 12 mo; during treatment, HBV-DNA levels remained above 2×10^4 IU/mL.

During treatment, a VBT was detected in nine patients, of whom two belonged to the 200 mg group (2/12; 16.6%) and seven to the 100 mg group (7/29; 24.1%). A BBT followed in all patients with VBT; in these patients, the ALT values were below 2 × the basal value in eight out of nine patients and in the range of a hepatitis flare in one patient (belonging to 100 mg group). At the end of the study, 63% of the patients in LAM-100 group and 83% in LAM-200 group were still VR (Table 2). None of the basal variables considered in Table 1 was associated with the outcome of the treatments.

In 11 patients who were constantly negative at the HBV-Monitor test, the RT-PCR revealed transient viremia blips below 80 IU/mL. This feature was recorded in seven out of the nine patients who subsequently presented a BT and in four out of the 32 patients with end of study response, the latter belonging to the 100 mg group (Table 3; $P = 0.001$).The negative predictive value and positive predictive value for BT were 0.93 and 0.63, respectively. Viremia blips occurred in 10 out of 29 (34.4%) patients who were initially responders to LAM 100 mg and in one out of 12 (8.4%) of those responding to LAM 200 mg. On the whole, the patients who showed absent or partial viral suppression (i.e., a PNR or a VBT or viral blips) were 17 out of 35 treated with LAM 100 mg and two out of 12 treated with LAM 200 mg ($P = 0.051$).

Lamivudine resistance mutations were researched in 13 cases, five of whom were PNRs and eight BTs (Table 4). None of the primary non responders had detectable mutations at baseline; however, all of them developed rtM204V/I mutant (none with concomitant rtL180M) while continuing LAM therapy, two with an ALT increase after the appearance of the mutation and one with a hepatitis-like flare. Among BT patients, seven presented rtM204V/I (four with concomitant rtL180M) and one patient presented an ALT flare with no detectable mutations. Mutations preceded the clinical BT by one to

Table 3 Viral blips observed during treatment. A viral blip is a transiently detectable viremia below 4×10^2 copies/mL in a responder patient

	Lam 100	Lam 200	Total
Primary responders (*n*)	29	12	41
Viral blips *n* (%)	10 (34.4)	1 (8.3)	11
Breakthroughs no.	7	2	9
Viral blips *n* (%)	6 (85.7)	1 (50)	7 (77.7)[a]
End of study responses (Amplicor)	22	10	32
Viral blips *n* (%)	4 (18.2)	0	4 (12.5)[a]

[a]Viral blips among patients with breakthroughs *vs* viral blips among patients with end of study response; 77.7% *vs* 12.5%; *P* = 0.001.

Table 4 Lamivudine resistance mutations and HBV genotypes in patients with a virological breakthrough or primary non-response

Patients	Genotype	Baseline	Mutation (mo)
Breakthrough			
[1]1	D	Wt	rtM204V (11)
[1]2	D	Wt	None (19)
[2]3	D	Wt	rtM204V (24)
[1]4	A	Wt	rtM204V rtL180M (28)
[1]5	D	Wt	rtM204V rtL180M (32)
[1]6	D	Wt	rtM204V rtL180M (19)
[1]7	D	Wt	rtM204V rtL180M (21)
[1]8	D	Wt	rtM204I (12)
Primary non-response			
[1]1	D	Wt	rtM204I (9)
[1]2	D	Wt	rtM204V (11)
[1]3	D	Wt	rtM204I (11)
[1]4	D	Wt	rtM204I (9)
[1]5	D	Wt	rtM204I (12)

Treatment group: [1]LAM:100; [2]LAM:200.

eight months. HBV genotypes were detected in 24 patients, all but one were genotype D.

Lamivudine was well tolerated; one patient belonging to the 100 mg group discontinued the treatment due to an increase in serum amylase to eight times the UNL. No serious adverse events were recorded in either treatment group.

DISCUSSION

This study was designed to check whether a long-term, double dose lamivudine treatment is feasible in patients with chronic hepatitis B and has the potential for a more profound and stable suppression of viral replication.

Basically, lamivudine pharmacokinetic is subjected to significant individual variability[20-22]. Plasma concentrations of lamivudine do not reflect its antiviral activity, since it depends on the 5-triphosphate anabolite of the drug, which is formed through an intracellular, saturable enzymatic process. Higher dosage of lamivudine produced higher intracellular lamivudine triphosphate concentrations. The intracellular half-life of active compound varies from 17 to 19 h in hepatic cell lines and from 10.5 to 15.5 h in peripheral blood mononuclear cells (PBMCs)[23,24] which suggested a dose interval of 12 h in HIV patients. In our study giving daily LAM 200 mg in two refracted doses may have enhanced intracellular active drug availability and even reached adequate anti-viral concentrations in extrahepatic sites of HBV replication.

In chronic hepatitis B, lamivudine doses ranging from 25 to 300 mg/d were used obtaining a steady viral inhibition at 100 mg[17,18] as measured by a hybridization assay. Looking at long term efficacy, Yuen *et al*[25] found no difference in the cumulative incidence of resistance mutations between patients treated with lamivudine 100 mg/d and those who received 25 mg/d for one to three years, though viral suppression was less effective at lamivudine 25 mg than at lamivudine 100 mg. This suggests that viral suppression may be sub-optimal even at the dose of 100 mg and is in keeping with our results at 100 and 200 mg.

In our study, the use of a sensitive PCR method highlighted that viral suppression was less efficient at 100 mg/d and this was the background for mutant selection and viral breakthroughs. Indeed, some patients classified as VR by Monitor assay had viremia blips below 80 IU/mL. This phenomenon was significantly associated with subsequent stable resurgence of viral replication and was mostly observed in patients receiving 100 mg lamivudine. At the end of the study a stable viral suppression was achieved in 83% of the patients receiving 200 mg LAM and in 51% of those treated at 100 mg/d.

There are some practical consequences from these observations, which are summarized in Figure 1. First of all, patients under treatment with lamivudine require strict follow-up with a high-sensitivity PCR assay, in order to detect, as soon as possible, even low level viremia. Secondly, an early change in therapeutic strategy is advisable for patients with viremia blips, by switching to adefovir or to entecavir[26-28], in order to prevent virological and biochemical BTs. Finally, patients with undetectable HBV-DNA and no viremia blips are candidates for continuing long-term lamivudine monotherapy, since the absence of viremia blips is predictive of long-term response.

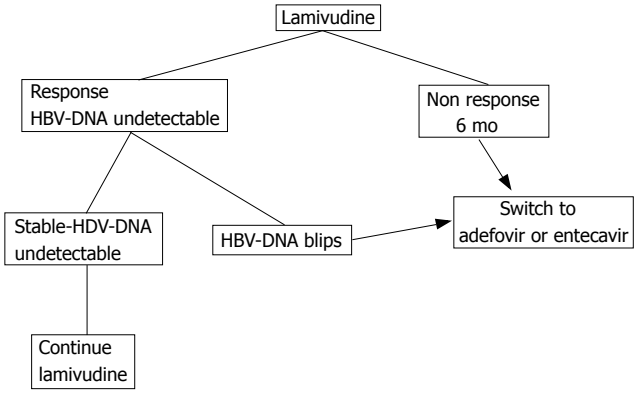

Figure 1 Flow-chart for patients under lamivudine treatment.

Genotype D of HBV was largely prevalent in our series and this raises the question as to whether the results are applicable to different HBV genotypes. There is evidence that response rates to Peg-interferon depend on HBV genotypes while less clear-cut results were obtained with anti-HBV analogs[1,29-31].

None of the primary non-response was associated with a pre-existing mutation, supporting the concept that the pharmacokinetic of the drug may play an important role in primary non-response and that higher drug doses may force this pharmacokinetic defect. Alternatively, PNR patients might harbour viral variants not detected by our method. Lamivudine resistance mutations were detected in all but one patient with virological BT.

In all primary non-responders continuing the treatment caused the appearance of resistance mutations, with further ALT elevation. From a practical point of view, patients who do not respond to a standard lamivudine dose should stop the treatment within the first six months (Figure 1), since a response after this period is unlikely. There is no data on early shifting to a higher lamivudine dose after an initial non-response to a standard dose.

In conclusion, our results indicate that the patients under lamivudine should be monitored using a high sensitivity PCR assay. An extended treatment with a double dose of lamivudine is feasible in chronic hepatitis B, and has the potential for a more pronounced viral suppression than a standard dose. Since lamivudine is a well-used, non-toxic and non-expensive anti-viral agent, these data should stimulate more powered studies aimed at optimizing treatment strategies.

COMMENTS

Background

Sustained viral suppression is the main goal of antiviral therapy in chronic hepatitis B. Lamivudine was the first antiviral drug approved for HBV treatment, at the standard daily dose of 100 mg. Although it was safe and potent, it caused the emergence of drug-resistant viral strains, at a rate of about 15% a year. At present, other antiviral drugs against HBV are available (adefovir, entecavir, telbivudine) that show a higher genetic barrier than lamivudine. Recent guidelines do not recommend the use of lamivudine as first line therapy in chronic hepatitis B.

Research frontiers

Thousands of patients with chronic hepatitis B are under lamivudine treatment.

Furthermore, lamivudine is the cheapest anti-HBV drug. After five years of continued treatment approximately 25% of the patients have an undetectable HBV-DNA. Can we improve the efficacy of lamivudine and predict the long-term response to treatment? There is a lack of studies with a higher than standard dosage of lamivudine in immunocompetent patients. This article examines the possibility of prolonged treatment with double daily dose of lamivudine (200 mg) and the predictive value of close monitoring of serum HBV-DNA using a highly sensitive real-time PCR.

Innovations and breakthroughs

Patients treated with lamivudine 200 mg/d achieved a more rapid primary response and a more profound and long-lasting viral suppression. In fact, the use of a sensitive PCR for HBV-DNA detection showed that transient low viremia levels ("viral blips") were more frequently detected in patients receiving lamivudine 100 mg and predicted a virological breakthrough and the emergence of lamivudine resistance mutations.

Applications

Patients under lamivudine treatment require a strict follow-up using a highly sensitive PCR. Real time PCR has a lower detection limit of 10 IU/mL and seems the best method for this purpose. Detecting even low level viremia during treatment may predict the emergence of resistance mutations. In this case, an early change in therapeutic strategy is advisable as indicated in Figure 1 of the paper. The paper suggests that therapy with double dose lamivudine is feasible and may achieve better results than the standard dose.

Terminology

Virological breakthrough is the increase of at least one log of the plasma viral concentration during anti-viral therapy. In most of the cases it is due to the emergence of viral resistant strains. Biochemical breakthrough is the increase of ALT value that follows the virological breakthrough. Genetic barrier may be defined as the probability of not reaching any resistant escape strains.

Peer review

The aim and content of the study were considered innovative. However, some limits come from the low number of patients enrolled in the 200 mg lamivudine group. As the authors stated, these results should encourage further trials.

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RAPID COMMUNICATION

Effect of sustained virological response on long-term clinical outcome in 113 patients with compensated hepatitis C-related cirrhosis treated by interferon alpha and ribavirin

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Abstract

AIM: To assess the long-term clinical benefit of sustained virological response (SVR) in patients with hepatitis C virus (HCV) cirrhosis treated by antiviral therapy using mostly ribavirin plus interferon either standard or pegylated.

METHODS: One hundred and thirteen patients with uncomplicated HCV biopsy-proven cirrhosis, treated by at least one course of antiviral treatment ⩾ 3 mo and followed ⩾ 30 mo were included. The occurrence of clinical events [hepatocellular carcinoma (HCC), decompensation and death] was compared in SVR and non SVR patients.

RESULTS: Seventy eight patients received bitherapy and 63 had repeat treatments. SVR was achieved in 37 patients (33%). During a mean follow-up of 7.7 years, clinical events occurred more frequently in non SVR than in SVR patients, with a significant difference for HCC (24/76 *vs* 1/37, $P = 0.01$). No SVR patient died while 20/76 non-SVR did ($P = 0.002$), mainly in relation to HCC (45%).

CONCLUSION: In patients with HCV-related cirrhosis, SVR is associated with a significant decrease in the incidence of HCC and mortality during a follow-up period of 7.7 years. This result is a strong argument to perform and repeat antiviral treatments in patients with compensated cirrhosis.

Key words: Hepatitis C; Cirrhosis; Interferon alpha

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INTRODUCTION

In patients with chronic liver diseases, the prognosis strongly depends on the extent of liver fibrosis, as life-threatening complications mainly occur in patients with cirrhosis. In chronic hepatitis C in particular, hepatocellular carcinoma (HCC) is observed only in case of cirrhosis or severe fibrosis (some of them presumably with under diagnosed cirrhosis due to sampling error of liver biopsy). In patients with compensated hepatitis C virus (HCV)-related cirrhosis, the annual incidence of HCC, decompensation and death reach around 3%, 4% and 3%, respectively[1-4] and the main cause of death is HCC[1].

Interferon-α was approved for use in chronic hepatitis C in 1991. During the past 15 years, subsequent improvements have included extension of therapy to 48 wk, the combination of interferon-α with ribavirin, and the use of pegylated interferon. These progresses have resulted in improvement in the sustained virological response (SVR) rate in patients included in randomized trials from less than 10% with the initially recommended 24-wk course of interferon-α monotherapy to as high as 50%-60% with the combination of peg interferon and ribavirin for 48 wk. In unselected patients, the percentage of SVR is expected to be lower particularly in patients with cirrhosis.

Most studies of antiviral therapy of HCV have been limited to 6 mo of follow-up after the end of treatment and have not included HCC, liver decompensation or death as end points. Therefore, studies of the effect of antiviral therapy on clinical outcome of HCV-cirrhosis have largely been retrospective analyses of therapeutic trials using interferon-α alone, focusing on patients with cirrhosis[4-11]. Only three randomized trials have assessed

this effect, giving conflicting results[12-15]. Recent meta-analysis suggested a slight, but significant preventive effect of standard interferon-α monotherapy on HCC occurrence in patients with HCV-cirrhosis, especially in those who achieve SVR, who intrinsically represent a small proportion of patients in these trials[16,17]. The influence of interferon-α on the incidence of decompensation or death in patients with HCV-cirrhosis was less studied and more controversial[4,8,10,12,14,18].

At present, bitherapy with standard then pegylated interferon plus ribavirin has created a new perspective for patients with HCV because of the higher rate of SVR reported[19,20]. The clinical long-term benefit of bitherapy with standard interferon-α has been recently assessed in Chinese patients with HCV-cirrhosis[21]. Such data are still not available in Western patients. Therefore, to assess the impact of achievement of a SVR on clinical long-term outcome of cirrhosis, we conducted a long-term, retrospective, bi-centre analysis of prospective collected data from a cohort of 113 French patients with histological proven HCV cirrhosis treated at least 3 mo by different regimens including ribavirin plus standard or pegylated interferon, and periodically followed and screened for HCC according to standardized criteria.

MATERIALS AND METHODS

Patients

We retrospectively selected all the consecutive patients followed-up in two French liver centres between 1989 and 2006 fulfilling the following criteria: (1) HCV-related cirrhosis defined by association of positive serum anti-HCV antibodies and RNA, with typical liver histology; (2) absence of complication before or at inclusion (Child-Pugh class A); (3) absence of HBV or HIV co infection (negative serum HBsAg and HIV antibodies); (4) daily alcohol consumption < 50 g; (5) absence of contra-indication to antiviral treatment, particularly platelet and polymorphonuclear counts ⩾ 80 000/mm^3 and 1500/mm^3, respectively; (6) at least a 3 mo course of antiviral treatment using standard or pegylated interferon with or without ribavirin, according to therapeutic advance over time and international guidelines; (7) a regular follow-up ⩾ 30 mo after the starting of the first treatment; (8) absence of HCC or even suspicious findings such as liver nodule or serum level of alpha-fetoprotein (AFP) above 50 ng/mL; (9) residence in France allowing regular follow up.

Assessment of Response to antiviral treatment

SVR was defined as undetectable serum HCV RNA 6-mo after discontinuation of the last treatment. Non responder patients to a first line treatment were retreated once or more each time it was possible with the same or a different regimen according to therapeutic advance over time. Serum HCV RNA was measured annually during follow-up and at the time of the last visit. According to final virological response, patients were separated into SVR and non SVR groups. Patients not fulfilling SVR criteria, including all patients who relapsed after the achievement of the end of treatment response, were classified as non-SVR.

Follow-up

All patients were prospectively followed-up according to the same schedule in both centres. Complete physical examination, standard biochemical tests, serum AFP determination and abdominal ultrasonography (US) were repeated every 6 mo, whatever the virological response. Baseline then annual endoscopy of the upper gastrointestinal tract allows assessment of the presence of gastrooesophageal varices. In case of significant endoscopic portal hypertension, prophylactic treatment (propranolol and/or endoscopic treatment) was started. The length of the study was calculated from the starting date of antiviral therapy and ended at death or at the last follow-up visit.

Endpoints

When a focal liver lesion or increased AFP levels were detected, tomodensitometry and, whenever possible, fine needle guided liver biopsy were performed. Diagnostic criteria for HCC were: (1) histological and (2) clinical, in patients with AFP value greater than 400 ng/mL and evidence of focal liver lesion at imaging techniques. After 2002, the HCC diagnosis was based on the guidelines of the European Association for the Study of the Liver[22].

Liver-related complications (ascites, upper gastro-intestinal bleeding, and hepatic encephalopathy) were considered an endpoint in all patients with or without the occurrence of HCC. In the subjects who developed HCC, liver complications were recorded only when they occurred before tumour development. Ascites was diagnosed by clinical examination and/or US detection. Porto-systemic encephalopathy was defined by clinical parameters. The source of gastro oesophageal bleeding was confirmed by endoscopy whenever possible.

Dates and causes of deaths were recorded. Liver transplantation was considered as liver-related death endpoint. Reference date was February 2006.

Statistical analysis

Continuous variables are reported as mean and standard deviation, and categorical variables as absolute and relative frequencies. The Mann-Whitney rank-sum test (M-W) and the Kruskal-Wallis nonparametric analysis of variance (K-W) were applied to compare the means. The associations between Non-SVR or SVR status and events in 2 × 2 cross tabulations were tested using Fisher's exact test. In case of larger cross tabulations, we tested the correlation by computing Pearson's Chi-square, or by computing either the exact probability value, or the Monte Carlo estimate of the exact probability value. Cumulative incidence curves of liver-related complications, HCC and mortality according to response to interferon treatment were plotted using the Kaplan-Meier method. The differences between groups were assessed using log-rank tests. Data was censored when individuals died, received a liver transplantation or were lost during follow-up. The variables which proved to be significant at univariate analysis were tested by the multivariate Cox proportional hazards regression model to assess their independent effect on the development of events during the follow-up. The

Table 1 Baseline characteristics of patients, as a whole and according to final virological response

Characteristics	All $n = 113$	SVR $n = 37$	Non SVR $n = 76$	P^1
Male gender (%)	69 (61.1%)	31 (83.8%)	38 (50.0%)	0.0005
Age (mean, yr)	54.1 ± 11.2	50.6 ± 11.1	55.8 ± 10.9	0.02
Alcohol (g/d)				0.4
0	71 (62.8%)	21 (56.8%)	50 (65.8%)	
< 20	20 (17.7%)	9 (24.3%)	11 (14.5%)	
⩾ 20	22 (19.5%)	7 (18.9%)	15 (19.7%)	
HCV genotype[2]				0.0001
1	58 (61.1%)	11 (35.5%)	47 (73.4%)	
2	10 (10.5%)	7 (22.6%)	3 (4.7%)	
3	15 (15.8%)	8 (25.8%)	7 (10.9%)	
4	8 (8.4%)	1 (3.2%)	7 (10.9%)	
5	2 (2.1%)	2 (6.5%)	0 (0.0%)	
6	2 (2.1%)	2 (6.5%)	0 (0.0%)	
Body mass index (kg/m^2)	25.9 ± 4.0	24.6 ± 3.8	26.5 ± 4.0	0.015
Platelets (10^3/mm^3)	149.4 ± 56.2	152.9 ± 50.1	147.9 ± 59.2	0.4
Serum albumin (g/L)	43.0 ± 5.0	45.4 ± 6.1	41.8 ± 4.0	0.001
Bilirubin (μmol/L)	14.6 ± 9.6	14.3 ± 7.5	14.8 ± 10.5	0.6
Prothrombin activity (%)	83.7 ± 13.3	84.5 ± 12.6	83.4 ± 13.8	0.7
AFP (ng/mL)	14.4 ± 25.2	8.7 ± 12.9	17.3 ± 29.2	0.008
AST (× ULN)	2.9 ± 2.0	2.4 ± 1.4	3.1 ± 2.3	0.2
ALT (× ULN)	3.1 ± 1.9	3.1 ± 1.9	3.1 ± 2.0	0.7

ULN: Upper limit of normal range; SVR: Sustained virological response. [1]Between SVR and Non-SVR groups; [2]Not determinate in 18 patients (15.9%).

Table 2 Duration of follow-up and characteristics of treatment according to final virological response

	All $n = 113$	SVR $n = 37$	Non SVR $n = 76$	P^1
Follow-up from the first liver biopsy (mean ± SD, yr)	8.2 ± 3.1	8.2 ± 2.7	8.2 ± 3.3	0.7
Follow-up from the beginning of first treatment (mean ± SD, yr)	7.7 ± 3.0	7.7 ± 2.6	7.6 ± 3.1	0.6
Number of treatment courses				0.25
1	50 (44.2%)	19 (51.4%)	31 (40.8%)	
2	42 (37.2%)	15 (40.5%)	27 (35.5%)	
3	19 (16.8%)	3 (8.1%)	16 (21.1%)	
4	2 (1.8%)	0 (0.0%)	2 (2.6%)	
Type of treatment				1.00
α-interferon monotherapy	35 (31.0%)	11 (29.7%)	24 (31.6%)	
Bitherapy	78 (69.0%)	26 (70.3%)	52 (68.4%)	
Type of bitherapy				< 0.0001
α-interferon + ribavirin	40 (51.3%)	22 (84.6%)	18 (34.6%)	
Peg-interferon + ribavirin	38 (48.7%)	4 (15.4%)	34 (65.4%)	
Total duration of treatment (mo)	15.3 ± 9.05	14.3 ± 6.4	15.8 ± 10.1	0.4

SVR: Sustained virological response. [1]*P* values were obtained between SVR and non SVR groups.

results were expressed as hazard ratio (HR) and their 95% confidence interval (CI). Data handling and analysis were performed with the Statistical Package for Social Sciences (SPSS 13.0; SPSS Inc., Chicago, IL) and STATXACT. All tests were 2-sided and $P < 0.05$ was considered to be statistically significant.

RESULTS

Patients and treatment regimens

One hundred thirteen patients fulfilled inclusion criteria (mean age: 54 ± 11 years, males: 61%, HCV genotype 1: 51%) (Table 1). As a whole, the mean (± SD) cumulated duration of treatment was 15.3 ± 9 (range: 3-42, median 12) mo. Treatment courses were performed 1, 2, 3 and 4 times in 50 (44.2%), 42 (37.2%), 19 (16.8%) and 2 (1.8%) patients respectively (Table 2). Interferon-ribavirin bitherapy was administered in 78 patients (69%), using pegylated interferon in 38 cases. SVR was obtained in 37 patients (33%). Nineteen out of 113 treated patients (16.8%) became SVR after the first treatment, 15/113 (13.3%) after the second, 3/113 (2.7%) after the third and 0/113 (0%) after the fourth treatment. Among the 37 SVR patients, 11 (29.7%) received only a monotherapy versus 24 (31.6%) in the 76 non SVR group ($P = 1.00$). Cumulated duration of antiviral treatment (14 mo ± 6 mo *vs* 16 mo ± 10 mo) did not significantly differ between SVR and non SVR patients ($P = 0.99$). There was no significant difference between the 2 groups regarding the number and the patterns of treatment (Table 2). Furthermore, there was no heterogeneity among the 2 centres in terms of duration and patterns of treatment.

The main patients' baseline characteristics according to final response are reported in Table 1. Compared to non SVR, SVR patients were more often males (84% *vs* 50%, $P = 0.0005$), younger (51 ± 11 *vs* 56 ± 11 years, $P = 0.02$), leaner (25 *vs* 26.5 kg/m^2, $P = 0.015$), less frequently infected with HCV genotype 1 (35.5% *vs* 73.4%, $P = 0.0001$), and had higher serum albumin levels (45 *vs* 42 g/L, $P = 0.001$) and lower AFP serum level (8.7 *vs* 17.3 ng/ mL, $P = 0.008$).

Follow-up and clinical events occurrence

The mean duration of follow-up was 7.7 ± 3.0 years from the beginning of the first treatment in the whole population. SVR and non SVR patients did not differ in term of follow-up (7.7 ± 2.6 *vs* 7.6 ± 3.1 years, $P = 0.6$) (Table 2).

During the follow-up, at least one severe clinical event (HCC or ascites or hepatic encephalopathy or gastrointestinal bleeding or death) occurred in 37 patients with a significant higher frequency in non-SVR (44.7% versus 8.1%, Hazard Ratio 6.3 (95% Confidence Interval 1.9-20.4), $P = 0.002$). Ascites, hepatic encephalopathy or gastrointestinal bleeding occurred in 11/76 non-SVR and in 3/37 SVR (Figure 1); HCC occurred in 24/76 non-SVR and in 1/37 SVR (Figure 2). The difference between the two groups was significant only for HCC [$P = 0.01$, Hazard Ratio 13.6 (95% Confidence Interval 1.8-100.5)], while there was no significant difference for the occurrence of other clinical events [ascites, hepatic encephalopathy or gastrointestinal bleeding: $P = 0.44$, Hazard Ratio 1.65 (95% Confidence Interval 0.46-5.9)] (Table 3). The diagnosis of HCC was assessed by histology in 9 patients (before June 2002) and according to guidelines of European Association for the Study of the Liver[22] in 16 patients (after July 2002). The median number of nodules was 1.5 (range, 1-8) and the median size 30 (range, 10-65) mm. The median times from the beginning of the first treatment until complications were not different in SVR and non-SVR: 6.99 years in SVR and 6.36 years in non-SVR for the occurrence of HCC ($P = 0.150$); 6.91 years in SVR and

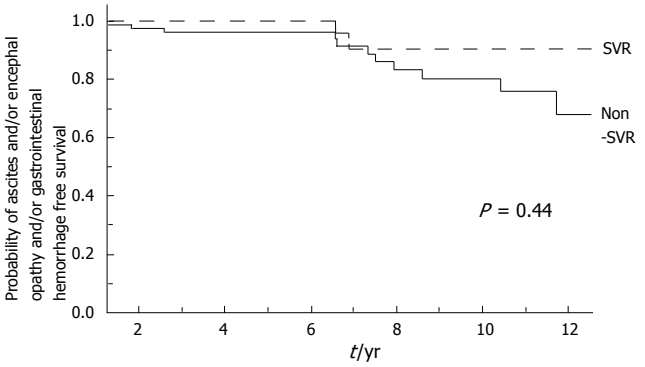

Figure 1 Incidence of ascites or encephalopathy or gastrointestinal haemorrhage from the beginning of the first treatment according to the response to antiviral treatment (SVR: Sustained virological response in dotted line; non-SVR in full line; Log-Rank, $P = 0.44$).

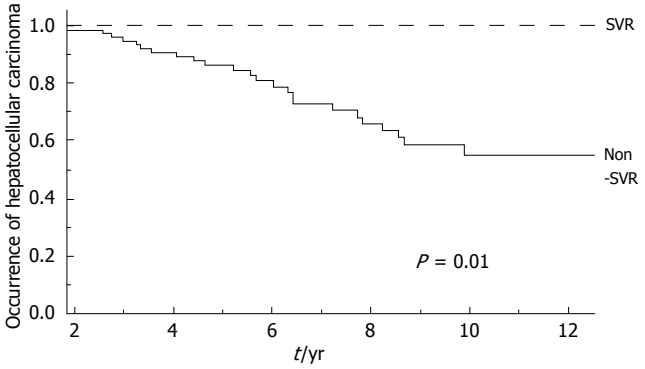

Figure 2 Incidence of hepatocellular carcinoma from the beginning of the first treatment according to response to antiviral treatment. SVR: Sustained virological response in dotted line; Non-SVR in full line; Log-Rank, $P = 0.01$.

6.78 years in non-SVR for the onset of decompensation or upper GI bleeding ($P = 0.531$). For the 37 patients who achieved a SVR, the median time from SVR (i.e., 6 mo after the end of the successful antiviral treatment) to the occurrence of complication was 4.79 years for HCC and 4.66 for decompensation. Death rate was significantly higher in case of non-SVR patients (20/76 *vs* 0/37; $P = 0.002$, Figure 3). Deaths were mainly related to liver disease and HCC was causative of 45% of the deaths. There was no heterogeneity among the 2 centres in terms of patterns of the duration of follow-up and of the number of events observed.

The multivariate analysis (Cox model) found two independent predictive factors for clinical events: SVR [$P = 0.001$, HR 7.1 (95% CI 2.2; 23.2)] and duration of treatment [$P = 0.001$, HR 0.93 (95% CI 0.89; 0.97)]. In other words, the Hazard for HCC was 7% lower for each additional year of treatment.

There was no significant difference with age, gender, or viral genotype for HCC occurrence.

DISCUSSION

Our study demonstrates a significant difference in long-term outcome between initially uncomplicated HCV cirrhotic patients with and without SVR after antiviral treatment. Over a mean follow-up period of 7.7 years, there were no deaths and virtually no severe clinical events in patients with SVR. At the opposite, HCC and liver decompensation occurred in patients without SVR with incidence rates similar to those previously reported in untreated cirrhotic patients[23]. We should emphasize that HCC was carefully screened before treatment, which eliminates HCC of small size appearing a few months after antiviral treatment.

Several studies performed in patients with chronic hepatitis C have suggested that successful antiviral treatment could result in a dramatic decrease in severe clinical events and mortality, life expectancy of patients with SVR being close to general population[6,9,10,24,25]. However, those studies used only interferon-α. In the past, interferon-α monotherapy resulted in a small rate of SVR in patients with cirrhosis due to low virological efficacy and poor tolerance resulting in a high rate of premature interruption of treatment. Accordingly, in the studies including a subset of cirrhotic patients, SVR rates in those populations did not usually exceed 15%.

Table 3 Severe liver-related events and deaths occurring from the beginning of first treatment according to final virological response

	SVR *n* = 37	Non SVR *n* = 76	*P*
Number of patients with at least one liver-related events or death during follow-up (%)	3[1] (8.1%)	34[2] (44.7%)	0.002
Number of patients (%) with			
HCC	1 (2.7%)	24 (31.6%)	0.01
Ascites	2 (5.4%)	8 (10.5%)	0.43
Digestive haemorrhage	1 (2.7%)	4 (5.3%)	0.62
Encephalopathy	0	2 (5.4%)	0.56
Number of deaths	0	20 (26.3%)	0.002
Liver-related	0	17	
Liver failure	0	7	
GI haemorrhage	0	1	
HCC	0	9	
Non liver-related	0	3	
Suicide	0	1	
Miscellaneous	0	2	

[1]1 patient had 2 complications; [2]4 patients had 2 complications. SVR: Sustained virological response; GI: Gastrointestinal.

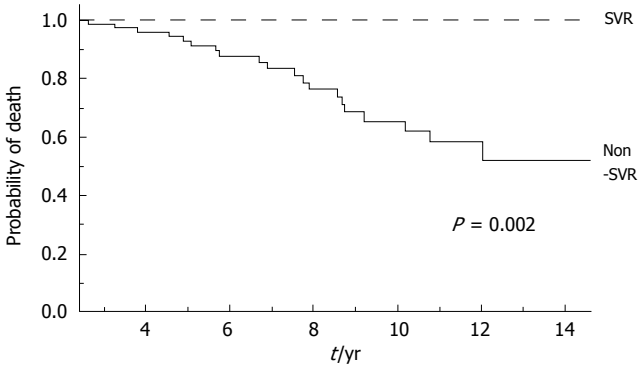

Figure 3 Incidence of death from the beginning of the first treatment according to response to antiviral treatment. SVR: Sustained virological response in dotted line; Non-SVR in full line; Log-Rank, $P = 0.002$.

Our study has several differences. Firstly, it was strictly restricted to patients with histological proven cirrhosis. This population is clearly at high risk of severe short-term complications occurrence as demonstrated by previous studies[1,26]. Secondly bitherapy using interferon-α and ribavirin was used in about 70% of cases in our study, with pegylated interferon in 38 cases. Thirdly antiviral treatment was repeated each time it was possible due to the absence of a priori contra-indication to treatment in those patients. Overall, after one (51.4%) or repeated (48.6%) antiviral treatments, 33% of our patients achieved HCV eradication, which is a rate twice higher than those obtained in previous studies.

Due to the lack of randomization, patients with SVR were obviously different from non SVR. Particularly, they were younger, more often men and significantly more frequently infected with HCV genotypes 2 or 3 than non SVR patients, as observed by Bruno *et al*[10]. Excepted male gender, these factors had been previously identified as predictive factors of SVR and it is not surprising to identify them in our study. The reduced rate of serious events in patients with SVR could result more from the presence of protective factors than from antiviral treatment. Patients with SVR were younger and this could result in a lower risk to develop complications as demonstrated for HCC occurrence. The role of genotype in spontaneous outcome of HCV-related cirrhosis although still debated does not seem to be a determinant factor in this setting and the male sex is a favouring factor of fibrosis progression and HCC occurrence. Subsequently the absence of serious event seems related to the complete response to treatment.

Several successive treatment courses have been performed in most of the patients, representing a cumulated time of treatment of 15.3 ± 9 mo. It could explain why clinical event incidence was decreased with a linear fashion from the start of treatment even if viral eradication was obtained later with further treatment. About half of SVR patients had no HCV eradication after the first course of treatment. It could seem questionable that severe events incidence have been reduced even before eradication was achieved in those patients. We postulate that it could reflect a more complete blockade of HCV replication in those patients occurring from the first course of treatment and due to higher sensitivity of virus. The fact that patients received similar duration of treatment in both groups is in favour of this hypothesis and against a non specific effect of treatment such as antifibrotic and/or antiproliferative effects of interferon.

The association between SVR and a significantly reduced number of clinical events has been previously reported[10,16,17,25]. However, this result cannot be applied to the whole population of patients with HCV-cirrhosis. In this study as in others, patients were selected and those with initial major contra-indication to treatment were excluded. These patients are probably the most severe with the higher risk of developing complications such as HCC. In addition, due to the selection of patients, the risk of death related to co morbidity was very low in our study.

Even in case of SVR, the periodic follow-up of cirrhotic patients should be recommended, particularly HCC screening as it is worth noting that the only HCC observed in SVR occurred 4.8 years after the achievement of viral clearance. In spite of a significant decrease in the incidence of HCC in case of SVR, several isolated cases of HCC were reported 4.5 to 6.6 years after the achievement of a SVR[27-30].

In conclusion, repeated antiviral therapy actually results in SVR in 33% of patients with HCV-cirrhosis. Virological cure seems to be associated with a strong decrease in the incidence of complications particularly HCC. These results are a strong argument to perform and repeat antiviral treatments in patients with compensated cirrhosis.

COMMENTS

Background

HCV cirrhosis is a life threatening disease with annual incidences of hepatocellular carcinoma (HCC), decompensation and death reaching around 3%, 4% and 3% respectively. The achievement of a sustained viral eradication (SVR) with the initially recommended 24-wk course of interferon-α monotherapy is associated with a slight preventive effect on HCC occurrence but its influence on the incidence of decompensation or death was less studied and more controversial.

Reasearch frontiers

Subsequent progresses in the field of HCV treatment (including extension of therapy to 48 wk, the combination of interferon-α with ribavirin, and the use of pegylated interferon) have created a new perspective for patients with HCV-cirrhosis because of the higher rate of SVR. Thus, the clinical long-term benefit of antiviral treatment in patients with HCV cirrhosis needed to be re-assessed.

Innovation and breakthroughs

Long-term effect of standard interferon-α plus ribavirin therapy on incidence of HCC in patients with HCV cirrhosis was recently studied in Chinese patients. Our results are the first in Western patients. In addition, death was a clinical end-point and we showed a significant reduced mortality in patients who achieved SVR.

Applications

While firm recommandations on antiviral treatment for patients with compensated HCV-cirrhosis was not made by international conferences, this is a strong argument to perform and repeat antiviral bitherapy in these patients to achieve SVR.

Terminology

Sustained virological response (SVR) is defined by the absence of detectable serum HCV RNA six months after the end of antiviral treatment and is associated with a durable viral eradication in most patients. Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and the first cause of death in patients with HCV-cirrhosis.

Peer review

The manuscript by El Barks *et al* demonstrates that antiviral treatment in patients with HCV-related cirrhosis decreases the incidence of HCC. The data provided argue for an antiviral treatment in HCV patients. The study was well performed and the data are clearly presented.

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RAPID COMMUNICATION

Pre-existing cirrhosis is associated with increased mortality of traumatic patients: Analysis of cases from a trauma center in East China

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Abstract

AIM: To determine the impact of cirrhosis on trauma patients and define the factors predicting death.

METHODS: The data on patients admitted to the trauma center from January 2000-2005 were studied retrospectively. The clinical variables were recorded and compared to identify the factors differentiating cirrhotic trauma survivors from non survivors. Child's classification criteria were derived from the reviewed charts of cirrhotic trauma patients to evaluate their predictive value in cirrhotic trauma. Trauma registry was also used to generate a trauma control group by matching for age, sex, abbreviated injury score (AIS) over the same period of time. The outcome variables compared were mortality rate, time of ICU and hospital stay. Results were expressed as mean ± SD. These data were analyzed by SPSS.11.0 statistical software. Univariate analysis was performed to identify significant medical factors for survivor and non survivors subjected to chi-square test. Fisher's exact test and Student's *t* test were performed to determine the statistical difference between cirrhotic and control groups. $P < 0.05$ was considered statistically significant.

RESULTS: Poor prognosis of traum patients was associated with one or more of the following findings: ascitcs, hyperbilirubinemia (more than 2 mg/dL), hypoalbuminemia (less than 3.5 mg/dL), and prolonged prothrombin time (more than 12.5 seconds). Although Child's classification was used to predict the outcome in cirrhotic patients undergoing portacaval shunt procedures, no significant difference was found in mortality rate as a function of Child's classification.

CONCLUSION: Cirrhosis is associated with a higher mortality, a longer time of ICU and hospital stay of trauma patients. It seems that treatment of trauma patients with pre-existing severe liver disease is a challenge to surgeons.

Key words: Pre-existing cirrhosis; Trauma; Mortality rate

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INTRODUCTION

Liver cirrhosis is the tenth leading cause of death[1]. Although cirrhosis-related deaths have decreased over the years, the impact of cirrhosis remains with approximately 30 000 deaths annually, mostly in Asian countries[2]. Cirrhotic patients often suffer from complications[3]. Cirrhosis impairs nutrition, alters response to stress, and affects the functions of other organ systems.

Trauma continues to be a major public health problem in the world. Due to the rapid economic development in China, the number of motor vehicles has increased tremendously in the past decade[4], leading to more deaths and injuries resulting from trauma. WHO predicts that trauma injuries will result in about 2.3 million deaths globally by 2020, becoming the third contributor to the global death[5]. Trauma not only causes a significant loss of life, but also results in loss of economic, medical, educational, and legal resources.

Trauma, in combination with cirrhosis in patients, brings about a unique challenge. Surgeons in trauma centers treat a variety of patients every day, but treatment of traumatized cirrhotic patients remains a challenge[6]. It has been shown that the mortality and morbidity rates increase in patients with cirrhosis undergoing elective or emergency surgery[7]. Also the degree of hepatic insufficiency is a prime factor for determining the outcome in these patients[8]. Despite the interest in the surgical outcome of cirrhotic patients, few reports are available on the outcome of cirrhotic patients after traumatic

injury. This study reviews and analyzes the data on trauma patients with pre-existing cirrhosis who were admitted to a trauma center in East China from 2000 to 2005.

MATERIALS AND METHODS

Data on more than 11 000 trauma patients (64 trauma patents with pre-existing liver cirrhosis) admitted to the Trauma Center of the First Affiliated Hospital, College of Medicine, Zhejiang University, China, from January 2000 to January 2005 were contained in their respective trauma registries. The data on patients under the ICDM-3 index for cirrhosis patients with chronic liver disease were analyzed. The trauma registry abstracts and medical records of all patients diagnosed as hepatic cirrhosis were reviewed. Diagnosis of hepatic cirrhosis was confirmed by the past medical history, clinical examination, operation findings, biopsy, and/or imaging.

The clinical variables were recorded and compared to identify the factors affecting prognosis of the patients and prolonging survival of the patients. In addition, evidence of hepatic insufficiency was evaluated by ascites, hyperbilirubinemia (more than 2 mg/dL), hypoalbuminemia (less than 3.5 mg/dL), elevated alkaline phosphatase (more than 125 st/L), serum glutamic oxaloacetic transaminase (SGOT more than 40 st/L), and/or prothrombin time (more than 12.5 s) detected at admission.

The trauma registry was also used to generate a trauma control group consisting of 86 patients by matching for age, sex, abbreviated injury score (AIS) over the same period of time. The AIS-85 scores were used because they permitted us to match patients with similar injuries. No cirrhosis or chronic liver failure was found in patients within the control group. The outcome variables compared were mortality rate, time of ICU and hospital stay.

Results were expressed as mean ± SD. The data were analyzed by SPSS.11.0 statistical software. As our sample size was too small to perform multivariate analysis, a univariate analysis was performed to identify the significant predictive factors. The data were subjected to chi-square test. To determine the statistical difference between the cirrhotic and control groups, we compared the mortality rates by Fisher's exact test. Parametric values of AIS, and time of hospital and ICU stay were compared by Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Sixty-four cirrhotic trauma patients (5.9 per 1000 trauma patients) admitted to the Trauma Center of the First Affiliated Hospital, College of Medicine, Zhejiang University were include in this study. These patients were diagnosed as cirrhosis before admission and during laparotomy for traumatic injury, respectively. The etiology of cirrhosis was related to HBV infection and alcohol in 62 and 1 patients, respectively and unidentified in 1 patient. The demographic and outcome data are listed in Table 1. Although the major causes for injury were motor vehicle accidents (MVA) (Table 2), 30.77% (4 of 13) of deaths were due to accidental fall. Other causes for injury included superficial abdominal stab wound, criminal assault. Seven patients had sustained blunt thoracic traumas including rib fracture, pulmonary contusion. Blunt abdominal trauma as evidenced by hemoperitoneum, splenic rupture, and/or liver laceration was the predominant injury in 9 patients. Pelvic fracture or limbic long-bone fracture occurred in 36 patients. Twenty-seven patients had injuries involving multiple sites. The remaining 37 patients had injuries involving a single site and two of them died (Table 3).

Table 1 Demographic and survival data on patients studied (48 males, 16 females)

(*n* = 40)	Range
Mean age = 52	31-84
Mean TS = 12	6-16
Mean ISS = 12	5-34
Surv = 87.5%	

Table 2 Mechanism of injury

Mechanism of injury	Total	Non survivors *n* (%)
Fall	13	4 (30.77)
MVA	48	3 (6.25)
Other	3	1 (33.33)

Table 3 Injury characteristics

Site	Total	Non survivors *n* (%)
Head	22	2 (9.09)
Thorax	7	2 (28.57)
Abdomen	9	3 (33.3)
Pelvis/Ext	26	1 (3.85)
Multiple	27	6 (22.22)
Single	37	2 (5.41)

The clinical or laboratory findings associated with hepatic insufficiency in this group of patients are outlined in Table 4. Ascites was confirmed during operation or radiological examination in 16 patients (6 of them died). An elevated prothrombin time of over 12.5 s was found in 15 patients (including 7 non survivors). Serum bilirubin exceeding 2 mg/dL was found in 15 patients at admission (6 of them died). The presence of any of these parameters was associated with a significant increase in mortality rate ($P < 0.05$). SGOT, alkaline phosphatase and hypoalbuminemia were elevated in many patients, but did not significantly affect their outcome.

Child's classification could predict the outcome of cirrhotic patients undergoing portacaval shunt procedures. In order to identify the predictive value of Child's classification, we retrospectively derived Child's classification criteria from the reviewed charts of cirrhotic trauma patients. The mortality of cirrhotic trauma patients according to the (retrospective) Child's classification is listed in Table 5, showing that 92.2% of our cirrhotic trauma patients corresponded to Child's class A or B. No

Table 4 Presence of HEPATIC insufficiency

Parameters of hepatic insufficiency	Total	Non survivors *n* (%)
Ascites	16	6 (37.50)[a]
SGOT > 40 μ/L	36	3 (8.33)
Alk phos. > 125 μ/L	32	3 (9.37)
Ser bili. > 2.0 md/dL	15	6 (40)[a]
Ser alb. 6 ≤ 3.0 md/dL	16	6 (37.5)[a]
PT > Control	15	7 (46.67)[a]

[a]$P < 0.05$ *vs* survivors.

Table 5 Effect of Child's classification on mortality in cirrhotic trauma patients *n* (%)

Child's classification	No. of patients (% total)	Mortality
Class A	44 (68.75)	5 (11.38)
Class B	15 (23.44)	2 (13.33)
Class C	5 (7.81)	1 (20)
Total	64 (100)	8

significant difference in mortality as a function of Child's classification was found ($P > 0.05$).

The second part of our study focused on comparing the trauma registry data between the cirrhotic trauma and control groups (Table 6). The cirrhotic trauma patients had a statistical ISS score similar to the control trauma patients. The time of ICU and hospital stay, and the mortality rate in the cirrhotic trauma group were greater than those in the control group ($P < 0.05$).

Table 6 Comparison of outcomes of trauma patients and cirrhotic trauma patients

	Cirrhosis	Control
Age (yr)	51.80 ± 13.01	48.70 ± 15.4
Percentage male	75%	76.11%
AIS	14.25 ± 8.31	13.67 ± 6.56
hospital stay	21.26 ± 5.61	8.21 ± 4.25[a]
length of ICU stay	11.24 ± 4.21	4.23 ± 1.36[a]
Mortality rate	12.53%	1.26%[a]

[a]$P < 0.05$ *vs* cirrhotic patients.

DISCUSSION

Hepatitis B is one of the most common infectious diseases in Asian countries[9], and about 10% of Chinese people have been infected with hepatitis B virus (HBV)[10]. It is estimated that about 350 million people worldwide are chronically infected with HBV, approximately 15%-40% of them are expected to develop cirrhosis and end-stage liver disease[11], which is frequently followed by hepatocellular necrosis. Cirrhosis, regardless of its etiology, inhibits the liver's response to injury. The predominant histological features are wide-spread fibrosis and nodule formation with loss of normal hepatic architecture. These changes are manifested clinically as hepatic failure and portal hypertension, the magnitude of which determines the course and prognosis of individual patients[12].

Although great progress in traumatology has been made, the number of traumatic casualties still increases[13]. In China, trauma and intoxication were the 9th, 7th and 4th leading cause of deaths in 1975, 1985 and 2000, respectively. More than 100 000 people die of traumatic injury and millions of people are injured each year in China. Furthermore, experts predict that the number of traumatic casualties will double in the 22nd century[14]. All these suggest that trauma in combination with cirrhosis is a challenge to Chinese doctors.

Adequate hepatic function is necessary in physiological response to surgery or traumatic injury[15]. The liver plays a vital role in protein synthesis, detoxification, and immune responses. In a patient subjected to surgical intervention for traumatic injury, any degree of hepatic insufficiency would diminish the liver's ability to carry out these vital metabolic functions[16]. Because of impaired cirrhotic reserves, a surgical or trauma cirrhotic patient would be at a great risk of developing complications and death may occur during the recovery period[17].

The increased risk of cirrhotic patients undergoing surgery has been well documented[18]. The Child's classification system has been used to define the surgical mortality in cirrhosis patients[19] and is moderately accurate in predicting mortality and complications of portacaval shunt surgery, but less predictive when it is applied to other types of surgery[20]. The Child's classification is mainly to classify the risk of cirrhotic patients undergoing portosystemic shunt surgery[21], showing that the degree of hepatic decompensation correlates with the rate of operative mortality in these patients. Furthermore, if therapeutic measures are taken to improve the clinical status and Child's class of cirrhotic patients before operation, the outcome of portosystemic shunting can be improved.

Child's classification criteria could not be used to classify these patients because nutritional status and response to therapy are unavailable[22]. Furthermore, changes in mental status at admission cannot be solely attributed to the etiology of cirrhosis[23]. However, hepatic insufficiency, as determined by the presence of ascites and/or elevated prothrombin time, is correlated with the outcome of these patients undergoing surgical intervention. Multivariate analysis revealed that cirrhotic trauma victims presenting with ascites, hyperbilirubinemia, or elevated prothrombin time exhibit a uniformly lower rate of survival independent of injury characteristics[24].

The impact of cirrhosis on trauma patients has recently been addressed[25]. Thirty percent of trauma patients with pre-existing liver disease have an increased risk of death and an increased time of hospital stay[26]. Tinkoff *et al*[27] have also tried to define the variables predicting the outcome of survivors and non survivors. The results of these studies suggest that trauma cirrhotic patients behave as cirrhotic patients requiring emergency surgery with similar stress and compensatory responses and that the mortality is directly related to the extent of injury.

In addition, hepatic insufficiency further diminishes survival, regardless of the injury sustained[28]. In the present study, cirrhosis was associated with a higher mortality, a longer time of ICU and hospital stay of trauma patients.

Factors predicting death are APACHE Ⅱ. ISS. RTS2,

the number of packed red blood cells transfused and organs injured, which are associated with the severity of injury[29]. Our study showed that liver insufficiency was positively associated with a poorer outcome. The lower survival and increased complication rates of cirrhotic trauma patients suggest that there is no "margin for error" in managing these patients. Thus, several management suggestions can be proposed for the improvement in cirrhotic patients with abdominal trauma[30]. It is critical to promptly diagnose and treat injuries in cirrhotic trauma patients. Since bleeding complications are frequent in cirrhotic patients, early and aggressive correction of coagulation parameters and hypothermia is crucial[31]. Poor nutrition is common in these patients and low albumin is different in survivors and non survivors. Therefore, early appropriate nutritional support should be provided. Solutions rich in branched-chain amino acids and low in aromatic amino acids can reduce hepatic encephalopathy and improve the outcome[32,33].

In conclusion, cirrhotic patients constitute a small subset of trauma patients admitted to our institution. Cirrhosis has a significantly independent adverse impact on survival of these patients. Treatment of trauma patients with severe pre-existing liver diseases remains a challenge to the surgeon.

COMMENTS

Background

Liver cirrhosis is the tenth leading cause of death. Although cirrhosis-related deaths have decreased, it leads to approximately 30 000 deaths annually, mostly in Asian countries. Trauma is a major public health problem in the world. Trauma in combination with cirrhosis brings about unique challenges and problems in patients. Surgeons in a trauma center treat a variety of patients every day, but treatment of trauma cirrhotic patients remains a challenge.

Research frontiers

Many reports have shown that mortality and morbidity rates are increased in patients with cirrhosis undergoing elective or emergency surgery. Also the degree of hepatic insufficiency is a prime factor for determining the outcome of these patients. Despite the interest in the outcome of surgical cirrhotic patients, few reports are available on their outcome.

Innovations and breakthroughs

The authors reviewed and analyzed the data on trauma patients with pre-existing cirrhosis admitted to a trauma center in East China from 2000 to 2005. This study showed that liver insufficiency was positively associated with a poorer outcome, suggesting that cirrhosis has a significantly independent adverse impact on survival of these patients.

Applications

Several management suggestions are proposed for the improvement in cirrhotic patients with abdominal trauma. It is critical to promptly diagnose and treat injuries for cirrhotic trauma patient. Since bleeding complications are frequent in cirrhotic patients, early and aggressive correction of coagulation parameters and hypothermia is crucial. Early appropriate nutritional support should be provided.

Terminology

Hepatic cirrhosis: a kind of pathological changes in liver. Hepatic cirrhosis is frequently followed by hepatocellular necrosis. Cirrhosis, regardless of its etiology, inhibits the liver's response to injury. The predominant histological features are wide-spread fibrosis and nodule formation with loss of normal hepatic architecture. These changes are manifested clinically as hepatic failure and portal hypertension, the magnitude of which determines the course and prognosis of individual patients.

Peer review

This is an interesting manuscript, showing that cirrhosis is associated with a higher mortality, a longer time of ICU and hospital stay in trauma patients. Treatment of trauma patients with severe pre-existing liver disease remains a challenge to the surgeon.

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CASE REPORT

Invasive amebiasis and ameboma formation presenting as a rectal mass: An uncommon case of malignant masquerade at a western medical center

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Abstract

A 54-year-old man presented with rectal pain and bleeding secondary to ulcerated, necrotic rectal and cecal masses that resembled colorectal carcinoma upon colonoscopy. These masses were later determined to be benign amebomas caused by invasive *Entamoeba histolytica*, which regressed completely with medical therapy. In Western countries, the occurrence of invasive protozoan infection with formation of amebomas is very rare and can mistakenly masquerade as a neoplasm. Not surprisingly, there have been very few cases reported of this clinical entity within the United States. Moreover, we report a patient that had an extremely rare occurrence of two synchronous lesions, one involving the rectum and the other situated in the cecum. We review the current literature on the pathogenesis of invasive *E. histolytica* infection and ameboma formation, as well as management of this rare disease entity at a western medical center.

Key words: Rectal ameboma; Invasive amebiasis; Ameboma; Amebic dysentery; *Entameoba histolytica*

Hardin RE, Ferzli GS, Zenilman ME, Gadangi PK, Bowne WB. Invasive amebiasis and ameboma formation presenting as a rectal mass: An uncommon case of malignant masquerade at a western medical center. *World J Gastroenterol* 2007; 13(42): 5659-5661

INTRODUCTION

Amebiasis is an infectious disease caused by the protozoan *Entamoeba histolytica*. Infection rarely, but potentially may evolve into invasive colitis and formation of ameboma, which can closely resemble colorectal carcinoma. In general, the clinical spectrum of colorectal amebiasis ranges from asymptomatic carrier to severe fulminant necrotizing colitis with bleeding and perforation[1]. As previously reported, focal ileocolonic intussusceptions from ameboma formation serving as the lead point, although rare, can occur[2].

The experience with invasive amebiasis is largely from endemic countries. From documentation dating back to 1933, to date 2970 cases of invasive amebiasis have been reported in the United States, mostly comprising recent immigrants from Mexico, Central and South America, plus Asians and Pacific Islanders[3]. With increasing patterns of immigration and travel in and out of the US, continued recognition of this disease is required. Therefore, the roles of the gastroenterologist and surgeon are to maintain a high index of clinical suspicion in patients at increased risk for this disease entity, and to distinguish this from other more common causes of gastrointestinal masses, as well as instituting appropriate therapy. In this report, diagnosis, pathogenesis and treatment of this rare clinical entity from a western medical center are reviewed.

CASE REPORT

A 54-year-old man of Middle Eastern decent, without significant medical history, presented to a community hospital complaining of rectal pain and bleeding for about 2 d. The patient described the pain as constant, without aggravating or alleviating factors. In addition, the patient complained of constipation that led to mild, crampy abdominal pain. He denied nausea, vomiting, diarrhea or fever. The patient also denied any history of weight loss. Pertinent to the patients' presentation was that he had no history of recent travel abroad, anoreceptive intercourse, or family history of carcinoma.

Physical examination revealed a healthy, well-nourished man who was normothermic with normal hemodynamic parameters. His abdominal examination was unremarkable: soft and non-tender, without any appreciable organomegaly.

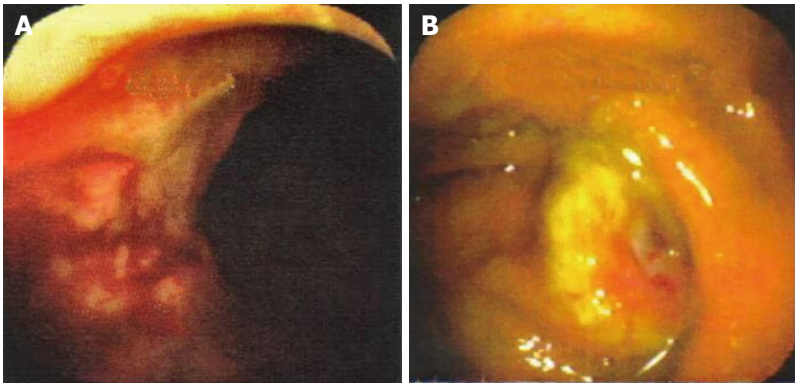

Figure 1 Endoscopy demonstrating ulcerated rectal (**A**) and cecal (**B**) lesions suggestive of carcinoma.

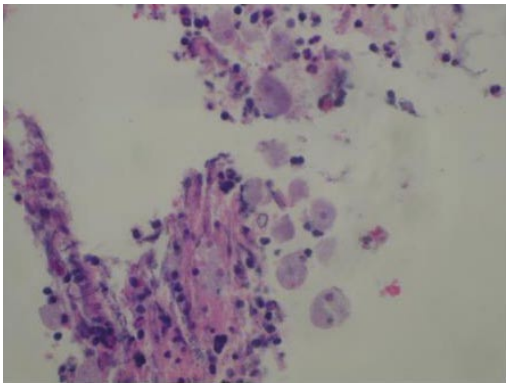

Figure 2 High-powered magnification of a hematoxylin-eosin preparation of a colonic biopsy that demonstrates trophozoites of *E. histolytica*.

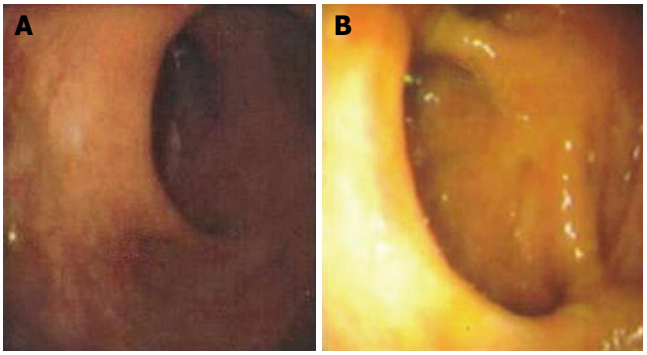

Figure 3 Follow-up colonoscopy subsequent to a completed course of antibiotic therapy, which demonstrates complete resolution of rectal and cecal lesions, with normal appearing colonic mucosa.

External anal inspection was normal. Rectal examination revealed a large, firm mass in the left lateral position, about 3 cm from the anal verge, with gross blood. Rigid proctosigmoidoscopy was performed, which revealed an ulcerated mass starting at the dentate line (Figure 1A). The surface of the lesion appeared necrotic and highly suggestive of carcinoma, which was the preliminary diagnosis. CT scan of the abdomen and pelvis revealed diffuse, non-specific thickening of the rectal wall without intra-abdominal pathology. A colonoscopy was performed that demonstrated an additional ulcerated lesion involving the cecum (Figure 1B). Both lesions were biopsied and were consistent with lymphocytic colitis. Importantly, protozoan organisms, consistent with amebiasis were identified (Figure 2). Serology for *Entamoeba histolytica* infection was also confirmed. The rectal and cecal lesions were determined to be amebomas as a result of invasive amebiasis. The patient was given a course of oral antibiotic therapy with metronidazole for 4 wk, with complete resolution of his symptoms. A follow-up surveillance colonoscopy and CT scan of the abdomen and pelvis demonstrated complete regression of both the rectal and cecal lesions (Figure 3A and B).

DISCUSSION

The intestinal protozoan parasite *E. histolytica* is the causative organism responsible for human amebiasis and amebic dysentery. Of epidemic proportions, it afflicts millions of people worldwide in developing countries, and about 40 000-100 000 people die annually from this disease[4]. Transmission is mostly by ingestion of contaminated food and water; however, venereal transmission *via* the fecal-oral route can occur[5].

Trophozoites are responsible for invasive disease and may lead to colonic mucosal ulceration. The gastrointestinal tract and liver are the two main organ systems affected by the parasite. Rarely, patients with long-standing infection develop ulcerative, exophytic, inflammatory masses that are indistinguishable from carcinomas and can become a considerable size, reportedly up to 15 cm in diameter[5]. Such lesions are referred to as amebomas. Moreover, colonic amebomas may present with synchronous amebic liver abscesses, which can also masquerade as advanced carcinoma of the gastrointestinal tract.

Amebomas result from the formation of annular colonic granulation tissue at single or multiple sites, usually within the cecum or ascending colon (Figure 4). Formation of an ameboma is an uncommon complication of invasive amebiasis. It occurs in 1.5% of all cases with invasive amebiasis[6]. Amebomas usually develop in the untreated or inadequately treated patients with amebiasis years after the attack of dysentery[5]. The current report is atypical, as our patient denied any history of antecedent symptoms suggestive of dysentery or foreign travel. Clinically, amebomas can also cause obstructive symptoms. Patients may also present with diarrhea or constipation and associated systemic symptoms, including weight loss

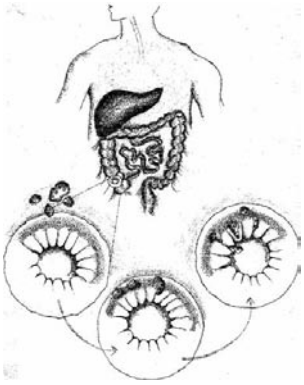

Figure 4 Pathogenesis of ameboma formation: infection is initiated by ingestion of fecally contaminated food or water containing *E. histolytica* cysts. Excystation occurs in the bowel lumen in which motile and potentially invasive trophozoites are formed. Invasion of the mucosa and submucosa may lead to colitis. Ameboma forms when severe inflammatory reaction occurs, with formation of granulation tissue that leads to a pseudotumor appearance.

and fever. In areas in which infection is prevalent, crampy abdominal pain plus a palpable mass usually suggests the diagnosis. In contrast, in western countries, a similar presentation alternatively raises suspicion of malignancy. The differential diagnosis for this clinical picture may also include inflammatory bowel disease (IBD), lymphoma, enterocolitis and intestinal tuberculosis[6]. In most cases in western countries, a diagnosis of colorectal carcinoma or IBD is considered more likely than a parasitic infection. Interestingly, there has been only a single *E. histolytica* outbreak in the United States, which occurred in Chicago in 1933 when sewage pipes contaminated tap water pipes in a hotel, which led to 800 cases of amebiasis[3].

The pathological range of amebic colitis extends from mucosal thickening with discrete ulcer formation, separated by regions of normal colonic mucosa, to diffusely inflamed and edematous mucosa. Severe forms of invasive amebiasis may progress to frank necrosis and perforation of the intestinal wall[3]. These findings are grossly similar to those found with IBD, in particular Crohn's colitis. Parasitic colitis results when the trophozoite penetrates the intestinal mucosal layer. Subsequent invasion occurs as a result of epithelial, neutrophil and lymphocyte cellular destruction by trophozoites[7].

The standard method for diagnosis of intestinal amebiasis is examination of stools by microscopy. However, the reported sensitivity of this method for identifying amebic organisms ranges from 25% to 60%[4]. False-positive results may also occur because *E. histolytica* is morphologically identical to non-pathological species, namely, *Entamoeba dispar* and *Entameba moshkovskii*[4]. Currently, more sensitive and specific methods, including antigen detection in stools and serum, and polymerase chain reaction techniques are now utilized[4].

The primary treatment for amoebic colitis is oral metronidazole therapy. Affected patients should be treated with metronidazole for 5-10 d. Longer intervals of therapy may be warranted in cases with ameboma formation, as in our patient. Treatment with metronidazole may be followed by a luminal agent such as paromomycin, iodoquinol or diloxanide furoate for 5-20 d to eradicate colonization[4]. Surgical intervention is rarely indicated except for rare instances of acute necrotizing colitis with bowel perforation, or if the patient fails to respond to anti-amebic therapy. Although amebiasis is a clinical entity rarely encountered in developed western countries, it should be included in the differential diagnosis of patients presenting with bloody diarrhea and a colon mass, with a history of dysentery along with travel to endemic areas. A high index of suspicion is required for appropriate diagnosis and treatment.

Unfortunately, there are no pathognomonic radiographic or endoscopic features suggestive of invasive ameboma formation; therefore, pathologic confirmation is crucial, as well as appropriate serology. Multiple biopsies may often be required to confirm the diagnosis. Two important caveats are essential to consider when managing patients with invasive amebomas: (1) follow up sigmoidoscopy or colonoscopy after completion of medical therapy to assure complete resolution of disease; and (2) persistent disease or incomplete regression should raise the suspicion for an underlying malignancy as the two entities may coexist[1].

In summary, the equivocal clinical symptoms (i.e., pain, bleeding and obstruction) that differentiate invasive amebiasis presenting as an ameboma from carcinoma may prove problematic. Notwithstanding, in a high-risk patient, clinical suspicion is warranted to assure appropriate diagnostic evaluation. Differentiating amebiasis from colorectal carcinoma by endoscopy with biopsy, serology, and other novel modes of antigen detection provides essential information. Therapeutic intervention with oral anti-parasitic medications rather than surgical management should then occur.

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CASE REPORT

Black esophagus with concomitant candidiasis developed after diabetic ketoacidosis

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Abstract

Black esophagus is a very rare disease and its pathogenesis has been unclear. Black esophagus developed concomitantly with candidiasis after diabetic ketoacidosis has not been reported yet. We report a case who developed esophageal stricture after the treatment of black esophagus and thus balloon dilatation was performed several times but failed, hence, surgical treatment was performed.

Key words: Esophagus; Candidiasis; Diabetic ketoacidosis; Surgery

Kim YH, Choi SY. Black esophagus with concomitant candidiasis developed after diabetic ketoacidosis. *World J Gastroenterol* 2007; 13(42): 5662-5663

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INTRODUCTION

Black esophagus was reported in 1990 by Goldenberg *et al*[1]. Black esophagus is a rare disease and defined as a dark, pigmented esophagus at endoscopy together with histologic mucosal necrosis. The etiology remains unknown, but is most likely multifactorial, even though most reports have suggested an ischemic pathogenesis[2-5]. It caused by diabetic ketoacidosis or candidiasis, but it is very rare etiology. Here, we present a case of a patient with black esophagus developed concomitantly with candidiasis after diabetic ketoacidosis.

CASE REPORT

A 34 year old male patient with the past history of type Ⅱ diabetes transferred to the emergency room with drowsy mentality. His general condition was poor. He presented with arterial hypotension (60/30 mmHg), hyperglycemia (819 mg/dL), electrolyte imbalance (sodium 118 mEq/L and potassium 6.5 mEq/L), impaired renal function (BUN 194 mg/dL and creatinine 2.90 mg/dL), and an acid-base disorder known as metabolic acidosis (pH = 7.241 with low bicarbonate levels). Urine ketones were elevated (+++). The clinical picture suggested that patient had diabetic ketoacidosis.

Two days after admission, hematemesis was detected, Esophago-gastro-duodenoscopy (EGD) showed black and friable mucosa from 3 to 4 cm below the cricopharyngeus to the cardia (Figure 1). Biopsies of the esophagus were not obtained at this time due to concern about possible perforation.

The patient gradually recovered after the conservative treatments such as intravenous proton pump inhibitor, total parenteral nutrition, oral intake restriction and antibiotics. Six days after admission, biopsies of the esophagus were obtained with EGD and revealed submucosal necrosis and candidiasis. After conservative treatments including antifungal agents (fluconazol 200 mg for seven days), the patient was discharged on the thirtieth day of admission. Subsequently, after 2 months, esophagus stricture was developed. Balloon dilation was performed 3 times during 6 months, but it was not improved (Figure 2A), and thus subtotal esophagectomy and esophagogastrostomy were performed (Figure 2B), and on the eighteenth day of surgery, he was discharged without complication.

DISCUSSION

Black esophagus was reported in 1990 by Goldenberg *et al*[1]. Black esophagus is a rare disease, and the incidence detected by EGD has been reported to be 0.01%-0.2%[2-5]. It is developed preferentially in the male (81%), and it has been reported to occur predominantly in the elderly[4].

The pathogenesis of black esophagus remains undefined and is probably a combination of an low systemic perfusion, gastric outlet obstruction, and malnutrition[2-5].

Several etiologies have been suggested including ischemia, lye ingestion, microbial infection related to a nasogastric tube, anticardiolipin antibody syndrome, herpes simplex esophagitis, diabetic ketoacidosis, severe vomiting, acute gastric outlet obstruction with massive gastroesophageal reflux, *Lactobacillus acidophilus* infection, acute caustic injury from detergent in the endoscope, antibiotics, Stevens-Johnson syndrome, and hypothermia[2,3,5]. Among 88 cases of black esophagus reported until now,

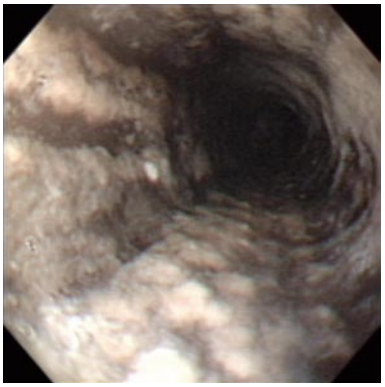

Figure 1 Endoscopy shows that esophagus is circumferentially black and friable.

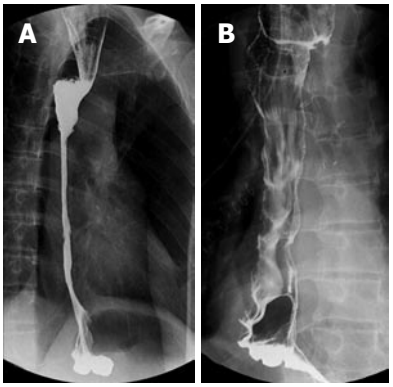

Figure 2 Barium swallow study. **A**: Stricture formation at middle esophagus; **B**: Improvement of the stricture was seen after surgical treatment.

cases whose etiology was diabetic ketoacidosis were 3 cases[4], cases whose etiology was fungal infection were 2 cases[5,6]; however, reports showing diabetic ketoacidosis and candidiasis concomitantly have not been reported.

Most patients are symptomatic, and the most common symptoms are upper gastrointestinal bleeding such as hematemesis, coffee-ground vomiting and melena[2-4].

The diagnosis is based on typical endoscopic appearance and on histopathological findings after the exclusion of true necrosis and ulceration. It shows the finding of the diffusely black esophageal mucosa, and in most cases it disappears suddenly in the Z-line. Histological findings of esophagus biopsy show necrotic debris, and it may show the finding of the necrotic mucosa without the viable epithelium, and occasionally, the submucosa and up to the muscularis propria, although rare, may be involved[2,4]. This case showed that submucosal involvement.

The prognosis of black esophagus is very poor, and its mortality reaches up to 31.8%-50%, although most of the deaths were caused by underlying illnesses[2-4]. Death secondary to esophageal necrosis occurs in less than 6% of cases[4]. Therapeutic modalities are not standardized, but most authors support a conservative approach by correcting the underlying disease, intravenous proton pump inhibitor, total parenteral nutrition, and oral intake restriction[2-5].

The major complications of black esophagus are esophageal stenosis and stricture formation, and they occur in 10.2%-15% of patients. Repeated dilation is required in most patients[2,4,5]. In our case, repeated dilation was not effective, and thus surgical treatment was performed. Other complications are esophageal perforation, mediastinitis and abscess formation, which occurs in approximately 6% patients[4].

Surgical treatments for black esophagus have been reported in 5 cases[4], nevertheless, all 5 cases were performed surgery for early complications such as esophageal perforation and our case is the first case of black esophagus performed surgical treatment for the late complication esophageal stricture.

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CASE REPORT

Giant submucosal lipoma located in the descending colon: A case report and review of the literature

Li Jiang, Li-Sheng Jiang, Fu-Yu Li, Hui Ye, Ning Li, Nan-Sheng Cheng, Yong Zhou

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Abstract

Colonic lipoma is an uncommon tumor of the gastrointestinal tract. Most cases are asymptomatic, with a small tumor size, and do not need any special treatment. However, we encountered one patient with a giant submucosal lipoma, with a maximum diameter of 8.5 cm, which exhibited symptoms such as intermittent lower abdominal pain, changes in bowel habits with passage of fresh blood and mucus per rectum, abdominal distension, anorexia and weight loss. Unfortunately, the possibility of colonic malignancy could not be precluded and left hemicolectomy was planned. The exact diagnosis of this special case was accomplished by intraoperative pathology. In the end, local resection was performed instead of left hemicolectomy. To the best of our knowledge, colonic lipoma exceeding 8 cm in diameter has not been previously reported. We, therefore, present this case and discuss age and sex factors, clinical and histopathological findings, diagnostic methods and treatment by reviewing the available literature, to serve as a reminder that colonic lipoma can also exist in patients with significant symptoms. In addition, intraoperative pathology should be investigated in those doubtful cases, so as to guide the exact diagnosis and treatment plan.

Key words: Colon; Lipoma; Age; Diagnosis; Therapy

Jiang L, Jiang LS, Li FY, Ye H, Li N, Cheng NS, Zhou Y. Giant submucosal lipoma located in the descending colon: A case report and review of the literature. *World J Gastroenterol* 2007; 13(42): 5664-5667

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INTRODUCTION

Lipoma of the colon is a rare condition which may be detected incidentally at colonoscopy, surgery or autopsy[1-3]. Most colonic lipomas are asymptomatic and need no treatment, whereas lesions exceeding 2 cm in diameter do produce symptoms[1,3-5]. Large colonic lipomas are usually mistaken for more serious pathology as a result of their rarity and variable presentation. Although the nosopoietic location of colonic lipoma usually varies with different cases, the commonest site is the ascending colon[2,4]. To the best of our knowledge, colonic lipoma exceeding 8 cm in diameter has not been reported previously. We report here, a patient with a giant lipoma, whose maximum diameter reached 8.5 cm, located in the submucosa of the descending colon, and review the literature regarding colonic lipoma.

CASE REPORT

A 42-year-old man presented to us in February 2007 with a 3-wk history of intermittent lower abdominal pain. One month previously, he experienced changes in bowel habits (7-10 times daily), with passing fresh blood and mucus per rectum, abdominal distension, anorexia and weight loss. A routine colonoscopy had been performed in another hospital 15 d previously, which revealed a yellowish, 7.5-cm diameter, spherical polypoid lesion arising from the lateral wall of the descending colon (Figure 1). The related biopsy revealed a lot of inflammatory necrotic tissue and a glandular cell with mildly atypical hyperplasia. Physical examination revealed mild tenderness in the left lower quadrant. Routine blood tests were normal except for mild anemia. Abdominal ultrasonography indicated a space-occupying mass of the lower abdomen. Considering the uncertain diagnosis, we suggested that colonoscopy or computed tomography (CT) should be performed again in our hospital, but this was refused by the patient. In view of his age, symptoms and related examinations, the possibility of colonic malignancy could not be precluded, and left hemicolectomy was subsequently planned.

At laparotomy, an 8.5 cm × 7 cm × 6.5 cm yellowish polypoid lesion, associated with numerous areas of ulceration on its surface, was seen arising from the descending colon. The lesion almost obstructed the whole lumen, and resulted in dilatation of the proximal colon. However, to our surprise, such a large lesion was only located on the submucosa, without invasion of the serosa. Subsequently, microscopical examination confirmed that this lesion was

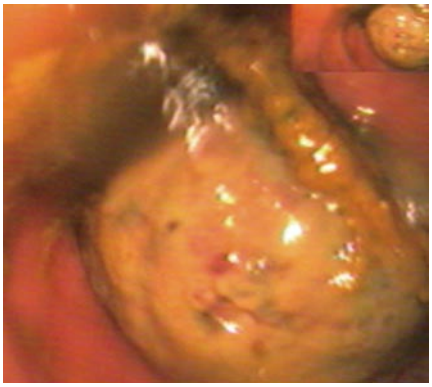

Figure 1 Colonoscopy showed a yellowish, spherical, polypoid lesion, with a lot of inflammatory necrotic tissue and numerous areas of ulceration on its surface, which arose from the lateral wall of the colon. The lesion almost obstructed the whole lumen.

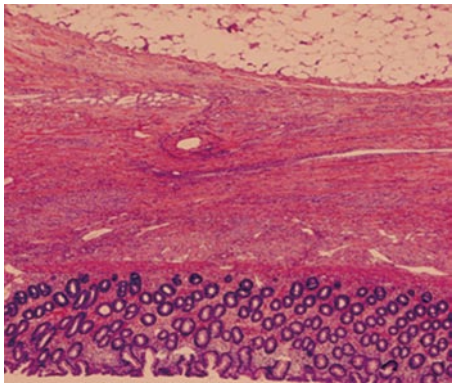

Figure 2 Histopathology showed that the lesion was located in the submucosa, of adipose origin, and was complicated with necrotic tissue and granulation on its surface (HE, × 100).

Table 1 Summary of the clinicopathologic features about treated lipomas in reviewed literature

	No	Sex		Mean age (yr)	Signs and symptoms				Pathology	Location				MMD (cm)
		M	F		AP	BPR	AIBH	anemia		AC	TC	DC	SC	
Ref.1	4	1	3	52	2	2	2	0	SL	1	0	1	2	2.5
Ref.2	3	1	2	51	3	0	0	0	SL	2	1	0	0	6.3
Ref.3	6	2	4	72.5	1	3	1	2	SL	3	0	0	3	2.9
Ref.4	8	4	4	53.3	3	3	1	1	SL (7) MSL (1)	6	1	0	1	3.7
Ref.6	5	1	4	62	3	5	1	0	SL	1	1	2	1	5
Ref.7	1	0	1	71	1	0	1	0	SL	1	0	0	0	5
Ref.8	3	0	3	71	0	3	0	0	SL (2) MSL (1)	1	0	0	2	2.2
Ref.9	2	1	1	56.5	0	1	1	0	SL	0	0	1	1	4
Present series	1	1	0	42	1	1	1	1	SL	0	0	1	0	8.5
Combined data	33	11	22	59.7	14	18	8	4	SL (31) MSL (2)	15	3	5	10	3.8
Percentage (%)		33.3	66.7		42.4	54.5	24.2	12.1	SL (93.9) MSL (6.1)	45.5	9.1	15.2	30.3	11.5

M: Male; F: Female; AP: Abdominal pain; BPR: Bleeding per rectum; AIBH: Alteration in bowel habits; AC: Ascending colon; TC: Transverse colon; DC: Descending colon; SC: Sigmoid colon; MMD: Mean maximum diameter; SSL: Simple submucosal lipoma; MSL: Multiple submucosal lipoma.

only located in the submucosa, and originated from adipose tissue, but it had necrotic tissue and ulcerations on its surface (Figure 2). Finally, the diagnosis was confirmed to be one of submucosal lipoma of the descending colon, and local resection was performed. The patient recovered and was discharged 12 d postoperatively.

DISCUSSION

Lipoma of the colon is an uncommon tumor of the gastrointestinal tract, and belongs to the group of benign non-epithelial tumors. As reported at autopsy, the incidence of colonic lipoma ranges from 0.035 to 4.4%[4]. In general, colonic lipomas do not cause symptoms and, therefore, are usually detected incidentally during colonoscopy, surgery and autopsy. However, a minority of lipomas can cause symptoms when the lesion is large, especially for those with a diameter > 2 cm[1,3-5]. To the best of our knowledge, colonic lipoma with a maximum diameter of 8.5 cm, associated with significant symptoms, has not been previously reported.

The clinicopathologic features of symptomatic lipomas are reviewed in the literature[1-4,6-9] and are summarized in Table 1. Thus, we can conclude that the most common signs and symptoms include abdominal pain (42.4%), bleeding per rectum (54.5%) and alteration in bowel habits (24.2%). With respect to sex distribution, there is a female predominance (66.7%), while other authors have found a nearly equal sex distribution[10]. The most common age is the fifth or sixth decades of life. As for its location, the most typical site for solitary colonic lipoma is the ascending colon (45.5%), whereas the lesion in our report was located in the descending colon. From Table 1, we can see that a solitary lesion is usually found in most cases; by contrast, multiple lesions occur in 6.1% of cases.

Microscopically, colonic lipomas are usually located in the submucosa, and numerous fibra intervals (Figure 3) can be observed in adipose tissue, resulting in the lobulated appearance of lipoma. Furthermore, varying degrees of fat necrosis, granulation and ulceration may be found on the surface of relatively large lipomas.

With the widespread application of colonoscopy, small lesions are found incidentally, and their diagnosis and treatment are mainly dependent on endoscopy[1-3]. However, large colonic lipomas are often mistaken for more serious pathology, as a result of their rarity and variable presentation. Therefore, more attention should be paid to how to increase the rate of preoperative diagnosis. Clinical fea-

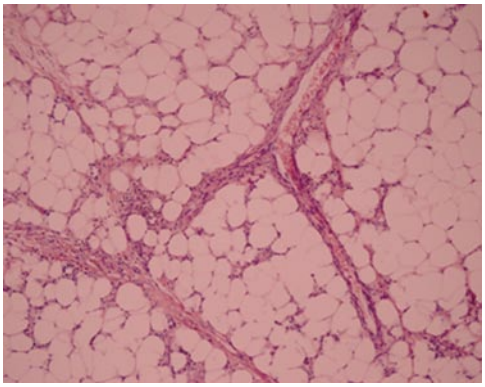

Figure 3 Histopathology showed the numerous fibra intervals in the adipose tissue (HE, × 200).

tures are still important, especially for those large lesions. Our patient with an 8.5 cm × 7 cm × 6.5 cm lesion should have presented with the appearance of complete intestinal obstruction. However, to our surprise, he did not present as an emergency with significant symptoms. Several factors may have contributed to this phenomenon. One potential explanation is the slow growth rate of colonic lipoma. Another, and perhaps more likely explanation is the long-standing obstruction caused by the lesion, which results in proximal colonic dilatation.

Although imaging findings may be less specific, they have still contributed to the preoperative diagnosis. Barium enema may demonstrate a filling defect, and the lesion may exhibit a lobulated appearance[4,6], but this phenomenon is non-specific and the lesion can be mistaken for another type of neoplasm, although water enemas are thought to improve the contrast with radiolucent fat[11]. For large colonic lipomas and acutely ill patients, CT and magnetic resonance imaging are the preferred methods because their imaging characteristics are relatively typical for adipose tissue, and they provide a rapid diagnosis[2,6].

Since most lipomas are submucosal, colonoscopy can provide direct visualization and pathologic examination via biopsy forceps. Thus, preoperative diagnosis mainly depends on colonoscopy. Typical lipomas appear as smooth, spheroidal, slightly yellowish polyps of variable size, with or without a pedicle[3]. In addition, three signs may contribute to the diagnosis, including "cushion sign"[12] (probing the polyp with a closed biopsy forceps will often yield a pillow-like indentation), "tenting effect"[12] (grasping the overlying mucosa with the biopsy forceps presents a tent-like appearance), and the "naked fat sign"[13] (biopsies may result in an extrusion of yellowish fat). Although colonoscopy is reliable for the diagnosis of the usual type of lipoma, it is more difficult for diagnosis of those lesions with an atypical, callous or ulcerated shape. In our case, the necrotic mucosa and numerous areas of ulceration that existed on the tumor surface, together with a relatively hard texture, may have confused the above-mentioned signs, therefore leading to the subsequent misdiagnosis. In addition, biopsy by colonoscopy may provide limited help for the diagnosis of some large lesions, because it cannot obtain adequate tissue. If the adipose tissue lies beneath the normal or ulcerated mucosa, it is not likely to be diagnostic; furthermore, if biopsy yields benign tissue, it is not possible to completely exclude the possibility of malignancy. The exact diagnosis still mainly relies on an intra- or postoperative pathology examination.

Many therapeutic interventions have been tried for the treatment of colonic lipoma, which have varied from hemicolectomy to segmental resection and local excision, according to the correct preoperative diagnosis and intraoperative findings. With the advancement of colonoscopy, endoscopic cautery snare resection of colonic lipomas has become popular and has been proven to be a safe therapeutic method, especially for small lesions[1,3,4,6,7,14]. However, various views with regard to endoscopic removal of large lipomas have been reported. Some studies have demonstrated that removal of lipomas $\geqslant$ 2 cm in diameter is associated with a greater risk of perforation[3,15,16]. On the contrary, some authors have reported that large pedunculated and large sessile lesions can be removed without perforation[6,7]. Kim *et al*[1] have performed endoscopic removal of lipoma with a maximum diameter of 3.8 cm, assisted by injection of saline solution with or without epinephrine into the submucosa beneath the lesion, with no complications. Bar-Meir *et al*[7] have described the safe endoscopic removal of a very large 5-cm lipoma. In addition, the feasibility of slow mechanical transection of a large colonic lipoma (4 cm) with an endoloop ligation technique has been demonstrated by Raju *et al*[15], whereas this novel technique may require application of additional loops several weeks later. The removal of colonic lipoma with the assistance of laparoscopy has also been reported[17].

However, on the basis of our case and the published literature, we think that surgical removal should be the preferred choice for the following indications: (1) lipoma with a diameter of > 4 cm, with a sessile appearance or limited pedicle; (2) unclear preoperative diagnosis; (3) lesions with significant symptoms, especially the appearance of intussusception; (4) involvement of the muscular layer or serosa; and (5) lesion cannot be resected radically under colonoscopy. Although colonic lipoma is a benign tumor, intraoperative frozen sections are required to ensure negative surgical margins, which can guide the choice of surgical approach. As far as our patient was concerned, left hemicolectomy would have been performed instead of local resection, if we had not taken a frozen section intraoperatively. Overall, intraoperative pathology is the most important examination for doubtful cases of colonic lipoma, which can also assist in guiding the exact diagnosis and treatment planning.

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symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
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cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
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Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
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www.bsg.org.uk/

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easl2007@easl.ch
www.easl.ch/liver-meeting/

Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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

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Issue with no volume

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

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Chapter in a book (list all authors)

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Author(s) and editor(s)

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

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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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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