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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007



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Recurrence of cholestatic liver disease after living donor liver transplantation

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further observations. The clinical course following LDLT may be affected by the genetic background shared between the recipient and the living related donor.

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Key words: Liver transplantation; Primary biliary cirrhosis; Primary sclerosing cholangitis; Living donor; Recurrence

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Abstract

End-stage liver disease, due to cholestatic liver diseases with an autoimmune background such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), is considered a good indication for liver transplantation. Excellent overall patient and graft outcomes, based mostly on the experience from deceased donor liver transplantation (DDLT), have been reported. Due to the limited number of organ donations from deceased donors in most Asian countries, living donor liver transplantation (LDLT) is the mainstream treatment for end-stage liver disease, including that resulting from PBC and PSC. Although the initial experiences with LDLT for PBC and PSC seem satisfactory or comparable to that with DDLT, some aspects, including the timing of transplantation, the risk of recurrent disease, and its long-term clinical implications, require further evaluation. Whether or not the long-term outcomes of LDLT from a biologically related donor are equivalent to that of DDLT requires

INTRODUCTION

Primary biliary cirrhosis (PBC) is characterized by the destruction of interlobular and septal bile ducts, leading to cholestasis and fibrosis^[1]. Primary sclerosing cholangitis (PSC) is also a chronic cholestatic liver disease, characterized by inflammatory and fibrotic bile duct lesions forming multiple strictures and dilatations of the intra- and extrahepatic bile ducts^[2-4].

Disordered immune regulation is considered to have a role in both PBC and PSC, though the specific immunologic mechanisms are yet to be clarified despite recent advancements^[2,5]. In both PBC and PSC, there is a gradual progression of cholestasis, which results in end-stage liver disease (ESLD). In PBC, the administration of ursodeoxycholic acid (UDCA) starting at an early stage of the disease improves the prognosis and is, therefore, recommended^[6,7]. On the other hand, effective medical treatment for PSC remains a matter of debate. UDCA administration, with or without the use of immunosuppressive agents, seems to have

a beneficial effect on liver function tests, assessed by blood chemistries. But, it is not clear whether there is a beneficial effect on delaying the progression of the disease and improving the overall survival rate^[8].

Liver transplantation is the optimal treatment for patients with PBC or PSC presenting with ESLD and associated complications, such as severe manifestations of portal hypertension^[5,8]. The short-term mortality rate, due to disease recurrence following transplantation, is lower in patients with PBC or PSC than in those with hepatitis C or hepatic malignancy. Longer follow-up, however, has revealed that both PBC and PSC can recur. But, the impact of a recurrence on long-term patient survival seems to be insignificant in deceased donor liver transplantation (DDLT)^[9].

Living donor liver transplantation (LDLT) has been accepted with greater enthusiasm in Asia, especially in the Far East where organs from deceased donors remain extremely scarce. Technical innovations driven by necessity have enabled the development of LDLT and extended its indications to adult populations^[10,11]. In November, 1993, the first successful LDLT for an adult recipient was performed at Shinshyu University by Makuuchi and colleagues^[12]. A 53-year-old woman with end-stage cholestatic liver disease due to PBC received a left liver graft from her son. The patient remains the longest survivor of this epoch-making success.

In LDLT, there is no waiting time. There is no competition between recipients for the availability of organs, unlike in DDLT. Liver transplantation becomes an option as soon as a socially-acceptable and medically-suitable dedicated living donor becomes available^[13]. Although a transplant with anatomically challenging features and smaller graft size may pose some difficulties, LDLT has some clear advantages when compared with DDLT. LDLT may be performed in a planned manner after thorough preparation at an earlier stage of the liver disease. In addition to the recipients' surgical risk, potential risks to the living donor cannot be overlooked. Transplantation should be considered when there is a clear survival benefit; that is, when the estimated risk of mortality for a patient on medical treatment alone exceeds that of the expected peri-operative mortality. We consider that LDLT becomes a beneficial option for patients with ESLD including that from PBC or PSC, who have a Model for End-stage Liver Disease (MELD) score of 15-17 (a calculated 3-mo mortality rate of approximately 5%), balancing the risk and benefit to the recipient and prospective living donor^[14]. LDLT for patients with co-existing hepatocellular carcinoma may be considered at an earlier stage.

PBC

Whether the combined symptoms of intractable pruritus, lethargy, or osteoporosis encountered in PBC should be considered an indication for LDLT in patients without significant signs of portal hypertension or liver failure remains an open question. At our institution, the indication of LDLT for PBC is based on the potential

for improvement or benefits in life expectancy. Unlike in DDLT^[15,16], less importance may be placed on quality of life in LDLT. We have considered that quality of life issues in the recipient do not outweigh even the slightest possibility of donor mortality or morbidity that may result in disability. In addition to the above-mentioned MELD score, the results obtained from the Mayo model are taken into account^[17]. Although the model was developed prior to the MELD era, and is not considered a factor for organ allocation in DDLT, we find that the Mayo model is very useful in the LDLT setting. As reported by Kim and colleagues^[18,19], there is a window of opportunity appropriate for LDLT, expressed as risk scores of 6 to 7.8. Risk scores higher than 10 are related to longer hospitalization after LDLT and mortality^[20,21]. When the risk of death by the natural disease course is predicted to outweigh the surgical risks of liver transplantation, LDLT may be planned and performed.

The 5-year survival rate after DDLT for PBC is approximately 80%^[18,22]. The anti-mitochondrial antibody (AMA) status does not change after liver transplantation and, therefore, is not diagnostic of recurrent disease after transplantation^[5]. PBC recurrence is currently defined according to the agreed criteria, including patient transplanted for PBC, persistence of AMA, and compatible liver biopsy^[23]. The largest single-center series with a mean follow-up period of 56 mo indicated a rate of such confirmed recurrence to be 17% at a mean follow-up period of 36 mo after transplantation. Interestingly, although histologically recognizable recurrence of the disease may be common, its effect on survival seems insignificant in DDLT^[22]. Liermann and colleagues^[15,22] demonstrated that patients with recurrent disease tended to be younger and receive organs from younger donors, and to have a longer warm ischemia time. But, such differences were considered to be clinically insignificant. The effect of a human leukocyte antigen (HLA) match between the donor and recipient remains unknown.

PBC is reported to recur after LDLT. Hashimoto and colleagues presented the first series of recurrent PBC after LDLT^[24]. All patients remained positive for AMA as in DDLT. The presence of mixed portal inflammatory infiltrates with granulomatous cholangitis was considered a definite histologic finding suggestive of recurrence. In 2 of 6 (33%) patients, such findings were established by protocol biopsy 1 to 2 years after LDLT. In the study, immunosuppression consisted of tacrolimus and steroids. Mycophenolate mofetil was not used. UDCA (600 mg/d) was administered to all patients.

The number of patients in Hashimoto's study^[24] is too small to draw a definitive conclusion. The high rate of recurrence (33% in 2 years) is in sharp contrast with that in an earlier DDLT series. As recently reviewed by Neuberger, early reports in DDLT in which the recurrence of PBC was evaluated in a smaller patient population and a shorter observation period indicated a tendency to underestimate the risk of disease recurrence, a finding that was later reversed by a larger series with a longer follow-up period^[15]. Compared to DDLT,

the history and volume of cases of LDLT for PBC is extremely limited. The rate of histologically confirmed recurrence at this very early stage is, therefore, somewhat disturbing.

Two single-center studies, both with 50 patients, were recently presented^[21,25]. Hasegawa and colleagues^[21] presented 3 and 5-year overall survival rates of 88% and 80%, respectively, with a median follow-up period of 35 mo. Before transplantation, AMA was positive in 42 patients (84%). AMA remained positive in 33 patients (66%) at 6 mo after transplantation. Multivariate analysis indicated that a lower updated Mayo risk score was a significant favorable factor for shorter hospitalization following LDLT, confirming the usefulness of the model in LDLT, as in DDLT^[21]. In the study, periodic liver biopsies were not performed, and occasional biopsy in cases of abnormal liver function did not present with findings suggestive of recurrent PBC. Biopsy-proven acute cellular rejection was observed in 18 (36%) patients. Because histologic findings are indispensable for the diagnosis of recurrent PBC, the study provides little information on disease recurrence, though the excellent midterm patient survival warrants further application of LDLT for PBC.

On the other hand, Morioka and colleagues^[25] presented 5-year overall survival rates of 67%. The recurrence of PBC was confirmed in 18% of patients within a median of 36 mo after LDLT (range 12-123 mo). The results of the study suggested that a lower number of HLA mismatches between donor and recipient, and a younger donor age resulted in better survival, though a lower number of HLA mismatches were also suggested to be a risk factor for PBC recurrence.

The study by Morioka and colleagues^[25] included ABO incompatible cases, which were not included in the study by Hasegawa and colleagues^[21], and recipients presented with higher MELD scores. The median MELD score in Hasegawa's study^[21] was 13, whereas that in Morioka's series^[25] was 23. Of the 50 patients, 14 (28%) died within 6 mo after LDLT in Morioka's series^[25], whereas 3 (6%) died within 6 mo after LDLT in Hasegawa study^[21]. It is not clear how the lower number of HLA mismatches affects short-term (6 mo) survival in the latter study. Recurrent PBC seems to be of less importance during this period as it was described to occur after 12 postoperative months. In fact, data more recently presented indicated that a simple comparison of HLA matching has little or no impact on survival^[26]. Clearly, the impact of HLA mismatches on PBC recurrence in LDLT requires further study.

The most recent registry study from Japan, in which 221 PBC patients were analyzed, reports a 5-year survival rate of 79%. Histologic evaluation was available for 70 patients, among whom 7 presented with findings compatible with recurrent disease with a median follow-up period of 36 mo^[27]. The information is limited, however, and it is, therefore, difficult to draw a universally acceptable conclusion on the overall long-term outcome of LDLT for PBC.

PSC

As for PBC, the indication for LDLT for PSC at our institution is based on the improved life expectancy. Although MELD score is considered more appropriate for DDLT, the new Mayo Model^[28] may be helpful for deciding the optimal timing of LDLT and preparation once a living donor candidate becomes available. Development of the model was based upon a large cohort of patients followed for two decades. Histologic evaluation by liver biopsy is not required and the score is easily obtained from readily available clinical variables. Its validity among the Far East Asian population, however, requires further analysis. At our institution, we currently consider a MELD score > 15 and/or a Mayo risk score > 2.0 as good starting points to prepare for LDLT.

PSC is considered to be a good indication for DDLT with an excellent 5-year graft survival rate of approximately 80%. A higher rate of re-transplantation compared to that for other indications (9.6% *vs* 4.9% within 2 years), however, has been recognized. But, its relation with recurrent disease is unclear. Recurrence of PSC after DDLT has been reported at rates between 1% to 33%, depending on the diagnostic criteria and follow-up duration^[8]. Graziadei and colleagues^[29] proposed combined cholangiographic and hepatic histologic criteria with strict exclusion criteria (cases with ABO incompatibility between donor and recipient, nonanastomotic strictures before posttransplantation day 90, anastomotic strictures alone, hepatic artery complications, or ductopenic rejection were excluded) and reported that PSC recurs after DDLT in 20% with typical radiologic manifestations found within a year, and histologic presentation within 3 years after DDLT. In their study, 5-year patient and graft survival rates were comparable between patients with recurrent PSC and patients with PSC without signs of recurrence.

A case suspected of recurrent PSC following LDLT was first reported by Kita *et al*^[30]. There have been sporadic case reports from Asian regions^[31-34], as well as from the West^[35-39], of LDLT for PSC from living related donors since then, but none reported recurrence; follow-up periods were less than 2 years in most case reports. Aside from the above case reports, cases of LDLT for PSC are found in moderate to large registries worldwide^[40-44]. Moon *et al*^[40] reported 2 cases of LDLT for PSC among their large series of 580 LDLTs. Soejima *et al*^[41] also reported 2 cases of LDLT for PSC in their series of 52 LDLTs. Both, however, lacked a specific description of the long-term outcome. The largest registry analysis by Maheshwari *et al*^[44] reported the outcome of 3309 PSC patients who underwent liver transplantation, among whom 69 underwent LDLT. Only 10 of those presented with a follow-up period longer than a year, however, and details regarding the outcome were not provided.

Thus, there is a significant lack of information specifically on the long-term outcome of LDLT for PSC, especially regarding recurrence. One explanation may be the rare incidence of PSC in the Far East

Table 1 Recurrence of PSC following LDLT

Series	No.	Rate of blood related donors	No. rec. PSC	Median period to dx. of rec.
Tokyo ^[47]	9	89% (5 out of 9)	4 (44%)	40 mo (range 14-66 mo) ²
Kyoto ^[48]	22	82% (23 out of 28 ¹)	13 (59%)	31 mo (range 22-71 mo) ³

No.: Number of patients; rec.: Recurrence; dx.: Diagnosis. ¹Twenty-eight patients underwent LDLT for PSC. Among them 22, (79%) survived for more than a year and were the subject of the analysis for recurrent PSC; ²Diagnosis based on radiologic findings. No protocol liver biopsies were performed; ³Diagnosis based on pathologic findings obtained from protocol liver biopsies and later confirmed by radiologic findings.

where LDLT is far more common than DDLT. Unlike in Nordic countries or in the United States, where PSC is one of the most important indications for liver transplantation^[45], this indication accounts for less than 3% of the total liver transplantations performed in Japan^[46].

Recently, two additional studies on LDLT for PSC were published from high volume centers in the region^[47,48]. The Tokyo group described the outcome of 9 adult LDLT cases, of which 8 were living-related. The median follow-up period was 3.5 years after LDLT. No ABO blood type-incompatible cases were performed. The outcome in terms of patient survival was satisfactory with a 5-year rate of 90%. When recurrence of PSC was evaluated according to Graziadei's criteria^[29], however, recurrent PSC was diagnosed in four patients. Rates of freedom from recurrent PSC at 1, 3, and 5 years were 100%, 73%, and 49%, respectively. The mean time to recurrence was 3.3 years. When limited to biologically related donor-recipient cases, recurrent PSC was diagnosed in 50% of cases. Interestingly, none of the patients presented with the HLA haplotypes associated with a higher susceptibility for developing PSC in the Caucasian population^[47].

The Kyoto group reported 28 patients with PSC who underwent LDLT. Among the 22 patients who survived for more than a year, 13 (59%) presented with PSC recurrence with a mean follow-up period of 31 mo, 5 of whom died or required re-transplantation for graft failure. The HLA haplotypes that may affect recurrence of the hepatic condition remain unclear, although HLA-DR15 is positively associated with ulcerative colitis. The group concluded that unlike PBC, the recurrence of PSC adversely affects the outcome in LDLT^[48]. Although there is a difference in the short-term mortality between the two reports, the high rate of recurrence reported in both studies requires further attention. Key features of the two series are summarized in Table 1. The clinical manifestations of recurrence described in both series seem more aggressive than those in patients that underwent LDLT for in PBC. The risk factors and susceptible genetic characteristics in LDLT remain unclear. Further prospective study with a protocol cholangiogram and genetic considerations with an HLA haplotype analysis is necessary.

CONCLUSION

LDLT provides a satisfactory short-to-midterm outcome for PBC and PSC. Both PBC and PSC, however, can recur after LDLT. The incidence of recurrence appears to be higher in LDLT compared to DDLT. The long-term impact of recurrence on survival, however, remains unknown at this point. PSC may present with a poorer long-term outcome, but further studies are necessary.

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TOPIC HIGHLIGHT

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Role of STAT3 in inflammatory bowel disease

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Abstract

Signal transducers and activators of transcription 3 (STAT3) play an important role in various autoimmune disorders including inflammatory bowel disease (IBD). Recent studies have revealed that STAT3 activation plays distinctly different roles between innate immune responses and acquired immune responses in colitis. STAT3-mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing the survival of pathogenic T cells. In contrast, STAT3-mediated activation of innate responses contributes to the suppression of colitis. This review will summarize the current understanding of the roles of STAT3 in IBD and the potential of targeting STAT3 for the treatment of IBD, emphasizing recent observations.

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Key words: Inflammatory bowel disease; STAT3; Inflammation; Innate immunity; Acquired immunity

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Sugimoto K. Role of STAT3 in inflammatory bowel disease.

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of idiopathic chronic relapsing immune-mediated inflammatory disorders, which is characterized by two major forms of intestinal inflammation: ulcerative colitis (UC) and Crohn's disease (CD)^[1]. Although the precise mechanisms of the pathogenesis of IBD still remain unclear, accumulating studies have shown that multiple cytokines, including interleukins (ILs) and interferons, are involved in the pathogenesis of IBD^[2,3]. Most of these cytokines activate various members of a family of cytoplasmic transcription factors such as signal transducers and activators of transcription (STATs)^[4,5]. Indeed, several recent studies have focused on the role of STATs on IBD. Among STATs, the role of STAT3 in IBD has been most well documented by recent studies obtained from human IBD studies^[6-8] and experimental IBD models^[9-12]. These studies have revealed that STAT3 is in a unique position for pathogenesis of IBD, because STAT3 plays distinct roles between innate immune cells and acquired immune cells. This review will focus on the roles of STAT3 in IBD from a different point of view, namely, innate immune systems *versus* acquired immune systems.

STAT3 AND IBD PATIENTS

STAT3 was initially identified as an acute phase response factor, an inducible DNA binding protein that binds to the IL-6 responsive element within the promoters of hepatic acute phase protein genes^[13]. Subsequent studies indicate that STAT3 becomes activated in response to a wide variety of cytokines and growth factors^[4,5,14]. After ligation of their specific receptors by these cytokines and growth factors, STAT3 is immediately phosphorylated, resulting in their dimerization and migration to the nucleus to induce several gene expressions encoding molecules that play a role in a variety of biological functions such as cell growth, anti- and pro-apoptosis, cell motility, negative feedback loop (suppression of cytokine productions), regulatory cytokine production and anti-bacterial activity depending on cell types^[4,5,14-17].

Although the role of STAT3 activation in the pathogenesis of IBD is still not very clear, the expression and activation of STAT3 protein in the mucosa of human IBD patients has been well studied. Lovato *et al* demonstrated constitutive activation of STAT3 in five out of seven CD patients in a small case series^[6]. Musso *et al* demonstrated that STAT3 activation was confined to actively inflamed colons from not only CD, but also UC patients^[7]. Accordingly, activated STAT3 was detected in isolated lamina propria mononuclear cells from inflamed IBD tissues, but not in peripheral blood mononuclear cells from control subjects or IBD patients. In this study, immunofluorescence demonstrated that the sources of activated STAT3 were macrophages and T lymphocytes, but not neutrophils. Mudter *et al* also showed that an increased amount of total STAT3 protein appeared in both UC and CD compared to noninflammatory control cells, and total STAT3 correlated with increased activated pSTAT3 in tissue sections from both UC and CD^[8]. They also showed that increased pSTAT3 also directly correlated with the histological degree of inflammation of tissues. Importantly, this activation is observed in both acquired cells such as T cells and innate cells such as colonic epithelial cells (CECs).

THE ROLE OF STAT3 ON ACQUIRED IMMUNE CELLS IN IBD

Since classical knockout of the *STAT3* gene in mice resulted in early embryonic lethality^[18], various cell type-specific STAT3 knockout mice were generated to elucidate the functional relevance of STAT3^[15,19-23]. Acquired immune cells, especially CD4⁺ T cells, have a considerable ability to induce the development and exacerbation of not only CD-like, but also UC-like experimental colitis^[24-27]. Importantly, Takeda *et al* showed that T cell specific STAT3 knockout mice showed impaired T cell proliferation^[19]. Moreover, they showed that STAT3 activation is involved in IL-6-dependent T cell proliferation through prevention of apoptosis. Atreya *et al* also showed that activation of IL-6/STAT3 cascade in lamina propria T cells can also induce prolonged survival of pathogenic T cells and, indeed, inactivation of this cascade contributes to the attenuation of chronic intestinal inflammation^[9]. These observations indicate that activation of STAT3 in the acquired cells plays a pathogenic role in chronic colitis by inducing prolonged survival of pathogenic T cells and disruption of immune tolerance. Indeed, inactivation of IL-6 or leptin/STAT3 cascades results in the suppression of acquired immune (T cell) mediated colitis^[9-12].

THE ROLE OF STAT3 ON INNATE IMMUNE CELLS IN IBD

Dysregulated host/microbial interactions have been postulated to play a crucial role in the development of IBD by inducing uncontrolled activation of acquired immune responses^[24,28-30]. Innate immune responses

mediated by CECs and phagocytic cells such as macrophages play a crucial role in the initial defense to maintain the appropriate host/microbial interactions^[15,22]. Accumulating studies have demonstrated that several mechanisms are involved in the maintenance and/or enhancement of the barrier function by CECs^[15,31,32]. Among these mechanisms, activation of STAT3 is considered to be an important pathway that can enhance and maintain not only the barrier function of epithelial cells, but also the activation of macrophage^[15,22]. Indeed, disruption of innate immune responses by genetically engineered either inactivation of STAT3 cascade in the CECs, or else macrophages induce intestinal inflammation^[15,21-23]. This observation is further supported by a recent study using a conditional STAT3 knockout mouse strain in which STAT3 deletion is induced during the adult life^[23]. This mouse strain shows efficient elimination of STAT3 protein in innate cells (macrophages and CECs), but not acquired cells (T and B cells), and develops a lethal colitis immediately after the deletion of STAT3 in the adult life. These findings indicate that, in contrast to acquired immune cells, activation of STAT3 in innate cells plays a regulatory role in colitis. Interestingly, inactivation of STAT3 in both CECs and macrophages results in lethal damage^[18,23], whereas such fatal inflammation is not observed in mice in which the inactivation is specifically induced in either CECs or macrophages^[15,22]. This raises a possibility that transient inactivation of STAT3 in both CEC and macrophage may cause lethal damage.

THE ROLE OF STAT3-ACTIVATORS ON IBD

Several cytokines and growth factors including IL-6, IL-7, IL-9, IL-10, IL-11, IL-15, IFN- α/β , leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), oncostatin M (OSM), leptin and growth hormone (GH) have been identified to activate STAT3 cascade with a different efficiency depending on the cell types^[4,5,14]. Among these, roles of IL-6, IL-11, IL-15, HGF, GH and leptin in experimental colitis have been demonstrated^[9-12,33-36]. For example, IL-6, IL-15 and leptin play a pathogenic role in colitis by activating STAT3 predominantly in the acquired immune cells^[9,10,12,36,37] (Figure 1).

In contrast, GH and IL-11, both of which activate STAT3 predominantly in CECs, contribute to the suppression of experimental colitis^[33-35]. Of note, human trials utilizing GH, IL-11 and anti-IL-6R mAb have been conducted and have shown that the strategies using these products are beneficial to IBD patients^[38-40] (Figure 1).

IL-22 has recently been shown to be preferentially expressed by the Th17 subset^[41,42] and has been demonstrated as a strong activator of STAT3^[43-45]. Recently, we have demonstrated that IL-22 contributes to rapid amelioration of local inflammation associated with a Th2-mediated colitis through activation of STAT3 in CECs^[46] (Figure 1). Since IL-22 specifically targets innate immune pathways^[16,45-47], selective activation of

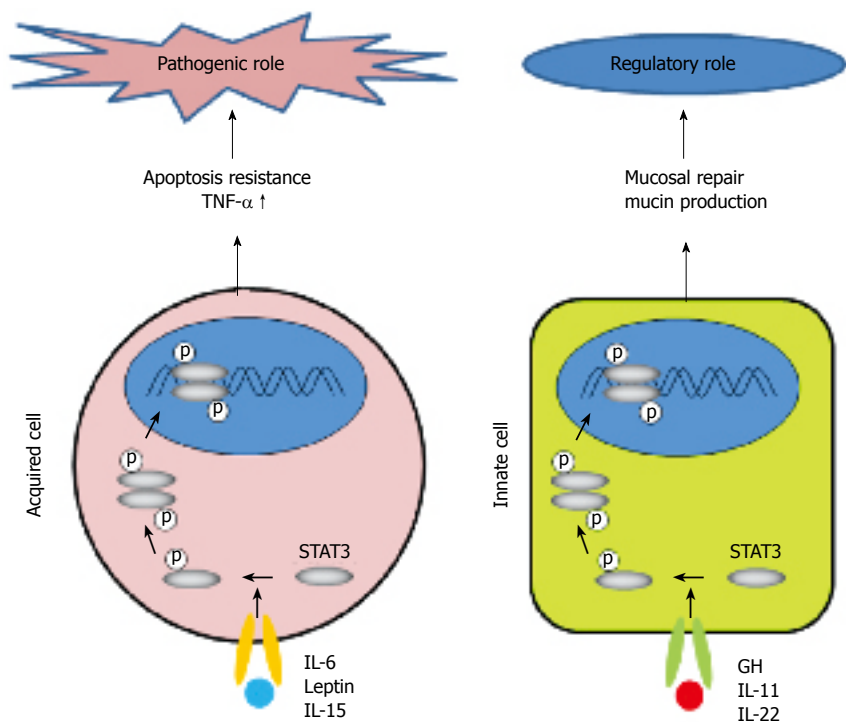


Figure 1 STAT3 plays distinct roles between innate immune cells and acquired immune cells. STAT3-mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing survival of pathogenic T cells and by inducing TNF- α . In contrast, STAT3-mediated activation of innate responses contributes to the suppression of colitis by enhancing the mucosal repair and by inducing mucin production.

	STAT3 activator	Target cell	Effect	Regulatory or pathogenic	Reference
Acquired	IL-6	CD4 ⁺ T cell	Apoptosis resistance	Pathogenic	9
	Leptin	IEL, LPMC	TNF- α \uparrow , MIP-1 α \uparrow , MIP-2 \uparrow	Pathogenic	12
	IL-15	CD8 ⁺ T cell	IFN- γ \uparrow , TNF- α \uparrow	Pathogenic	36
Innate	GH	CEC	Mucosal repair	Regulatory	33
	IL-11	CEC	Crypt cell proliferation after injury	Regulatory	40
	IL-22	CEC	Mucin production	Regulatory	46

STAT3 in CECs, but not acquired immune cells by IL-22 may contribute to amelioration or chronic Th2-mediated colitis.

These findings suggest that it is possible to activate or inactivate STAT3 in specific cell populations for the treatment of human IBD.

THE ROLE OF MOLECULES INDUCED BY STAT3 ON IBD

STAT3 has been shown to induce anti-apoptotic genes such as *bcl-2* and *bcl-xl* in T cells, and increase to resistance of lamina propria pathogenic T cells to apoptosis^[9]. In contrast, STAT3 can induce several molecules involved in the regulation of inflammation in CECs. Recent studies have demonstrated that STAT3 is required for the induction of IL-10, a well-known regulatory cytokine that is involved in the suppression of several inflammatory processes^[48] including experimental colitis^[15,49,50]. Local, but not systemic administration of this cytokine is of benefit in human IBD^[51,52]. Impaired IL-10 production in macrophage by the STAT3 deletion is involved in the development of colitis^[15], and enhanced production of this cytokine by CEC contributes to the suppression of colitis^[53]. Importantly, IL-22, a strong activator of STAT3, has been shown to induce IL-10 production by CEC lines^[45].

In addition to IL-10, STAT3 activation can also induce the expression of suppressors of cytokine signaling (SOCS) 3 that has been shown to play an important role in the suppression of acute colitis^[11,17]. Furthermore, STAT3 has been recently demonstrated to induce trefoil factors that regulate intestinal epithelial homeostasis^[22]. Moreover, IL-22-mediated STAT3 activation contributes to the improvement of colitis-associated mucus layer destruction associated with goblet cell depletion by enhancing the production of membrane-bound mucins (MUC1, -3, -10, and -13)^[46]. Membrane-bound mucins form a static external barrier at the epithelial surface and are stored in goblet cell vacuoles^[54-56]. Therefore, our recent study suggests that the enhanced mucus barrier formation participates in the IL-22/STAT3-mediated attenuation of Th2-mediated colitis^[46].

CONCLUSION

Recent data indicate that STAT3 is one of the crucial targets for the treatment of IBD. However, as the receptors of these cytokines and growth factors are present on both innate and acquired cells, activation of STAT3 is likely to occur in both cell types^[5,14,34-36,57,58]. Therefore, as the function of STAT3 is a double-edged sword, careful attention should be directed toward the cell population that is being targeted when one contemplates STAT3 inhibition or activation in human IBD^[59].

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New serological biomarkers of inflammatory bowel disease

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Abstract

Serological biomarkers in inflammatory bowel disease (IBD) are a rapidly expanding list of non-invasive tests for objective assessments of disease activity, early diagnosis, prognosis evaluation and surveillance. This review summarizes both old and new biomarkers in IBD, but focuses on the development and characterization of new serological biomarkers (identified since 2007). These include five new anti-glycan antibodies, anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), anti-manobioside IgG (AMCA), and antibodies against chemically synthesized (Σ) two major oligomannose epitopes, Man α -1,3 Man α -1,2 Man (Σ Man3) and Man α -1,3 Man α -1,2 Man α -1,2 Man (Σ Man4). These new biomarkers serve as valuable complementary tools to existing biomarkers not only in differentiating Crohn's disease (CD), ulcerative colitis (UC), normal and other non-IBD gut diseases, but also in predicting disease involvement (ileum vs colon), IBD risk (as subclinical biomarkers), and disease course (risk of complication and surgery). Interestingly, the prevalence of the antiglycan antibodies, including anti-Saccharomyces cerevisiae antibodies (ASCA), ALCA and AMCA, was found to be associated with single nucleotide polymorphisms (SNPs) of IBD susceptible genes such as NOD2/CARD15, NOD1/CARD4, toll-like

receptors (TLR) 2 and 4, and β -defensin-1. Furthermore, a gene dosage effect was observed: anti-glycan positivity became more frequent as the number of NOD2/CARD15 SNPs increased. Other new serum/plasma IBD biomarkers reviewed include ubiquitination factor E4A (UBE4A), CXCL16 (a chemokine), resistin, and apolipoprotein A-IV. This review also discusses the most recent studies in IBD biomarker discovery by the application of new technologies such as proteomics, fourier transform near-infrared spectroscopy, and multiplex enzyme-linked immunosorbent assay (ELISA)'s (with an emphasis on cytokine/chemokine profiling). Finally, the prospects of developing more clinically useful novel diagnostic algorithms by incorporating new technologies in serological biomarker profiling and integrating multiple biomarkers with bioinformatics analysis/modeling are also discussed.

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Key words: Serological biomarkers; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Anti-chitobioside IgA; Anti-laminaribioside IgG; Anti-manobioside IgG; Anti-synthetic mannoside antibodies; Multiplex enzyme-linked immunosorbent assay; Proteomics

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INTRODUCTION

Biomarkers of inflammatory bowel disease (IBD) are measurable substances in body fluids [such as blood (serological)], stool, or other parts of the body, as tools for disease diagnosis and/or prognosis. Application of IBD biomarkers is cheaper, less laborious, less invasive, and more objective compared to the endoscopy/biopsy-based approach^[1]. Current IBD biomarkers include serological, fecal and genetically predisposed gene polymorphisms^[2-5], as well as imaging biomarkers in conjunction

with imaging technologies such as optical, ultrasound, magnetic resonance imaging (MRI), X-ray, computer tomography (CT), position emission tomography (PET), single photon emission computed tomography (SPECT)^[6-9]. Among those, fecal^[10,11] and serological biomarkers, including systemic level of specific antibodies and other serum proteins^[4,12-14], have been most widely explored and/or used in clinical studies. However, none of the current commercially available biomarker tests/assays, including all of those mentioned in this highlight, can be used as stand-alone tools in clinics and, therefore, can only recommended as an adjunct to endoscopy in diagnosis, and prognosis of the disease^[1,15]. Considering that endoscopy is a highly resource-intensive process (involving frequent invasive, labor-intensive and expensive colonoscopic procedures), new IBD biomarkers and more comprehensive bioinformatic algorithms with multiple biomarkers are in great need.

The focus of this highlight is on new serological IBD biomarkers. However, the remarkable rapid development of IBD biomarkers in the last decade have made it impossible to separate the “old” from the “new”, since multiple biomarkers, both old and new, are being integrated in clinical studies. For example, 5 serum biomarkers, including ASCA, pANCA, anti-OmpC, anti-Cbir and anti-I2 (see below), were the most widely studied in the past decade, but are still being characterized and validated for their clinical utility. Since the status of these biomarkers has been reviewed extensively elsewhere^[4,12-14], they will be listed as “old” markers and only briefly reviewed, along with many other serological biomarkers reported before 2007. Anti-glycan antibodies, a newer panel of serum biomarkers, first reported in 2006^[16,17] and being validated since 2007, will be one of the major “new” biomarkers in this highlight. Serum cytokines, which are among the earliest inflammatory mediators studied, but are yet to be recognized as useful IBD biomarkers, will be reviewed and discussed. The prospects of developing new serological IBD biomarkers and integrating existing ones will also be discussed, particularly regarding the application of novel molecular approaches and proteomic technologies in biomarker screening and identification, as well as novel bioinformatic analyses of clinical utilities of multiple biomarkers.

“OLD” SEROLOGICAL IBD BIOMARKERS: A BRIEF OVERVIEW

Anti-Saccharomyces cerevisiae antibodies (ASCA) and perinuclear antineutrophil cytoplasmic antibodies (pANCA) were the first extensively characterized serological IBD markers^[18,19]. ASCA is more associated with Crohn’s disease (CD) while pANCA is more associated with ulcerative colitis (UC)^[4,13,20,21]. Three additional serum biomarkers were introduced later, including antibodies against outer membrane porin C (anti-OmpC), Pseudomonas fluorescens bacterial sequence I2 (anti-I2), and bacterial flagellin (anti-CBir 1)^[12,13,22,23]. These are five most extensively studied

serological biomarkers to date. Although exact data from independent studies vary, combinations of more than one of the 5 serological markers have been shown to have the most clinical value (see reviews^[4,12-14,24]). For example, ASCA and pANCA together have a specificity of approximately 90% for both CD and UC^[21,25-27]. These markers have been demonstrated as not only being useful for differentiating IBD *vs* healthy control or CD *vs* UC, but also as potential indicators and/or predictors for disease activity/location, disease course/complication, need for surgery, and prognosis of therapy. For example, CD patients who are positive in multiple anti-microbial antibodies (ASCA, anti-OmpC, anti-CBir, and anti-I2) have increased risk of having more complicated disease. Patients who are positive in all four of these biomarkers have 11-fold increased risk to develop penetrating and/or stricturing disease^[28-32]. CD patients positive with three markers (anti-OmpC, anti-CBir, and anti-I2) are more likely to have small bowel surgery than those who were negative (72% *vs* 23%). No similar association of serotype was found with disease phenotype of UC^[32].

Elevated levels of serological biomarkers were shown to be associated with IBD-susceptible gene variants. Family members of CD patients with NOD2/CARD15 3020insC variant was reported to have increased intestinal permeability, which has been positively associated with elevated serological biomarkers^[33,34]. However, reports on this relationship have been inconsistent^[35-38], even though more studies presented a positive association between serological biomarkers and susceptible gene variants^[32,37,38]. Future studies by independent groups with larger cohorts, well-defined clinical characteristics and patient populations (such as ethnicity) are necessary to resolve this discrepancy.

Other note-worthy aspects of these serological biomarkers include their potential value as subclinical biomarkers and their inherent geographic/ethnic heterogeneity. (1) Independent studies have shown that the prevalence of ASCA positivity is significantly higher (20%-25%) in unaffected first-degree relatives of patients with CD^[18,39] compared to general healthy populations (0%-10%), indicating a familial association. A much stronger indication that ASCA may be a potential subclinical biomarkers for CD came in 2005. In a serological analysis of a large serum depository, Israeli *et al* reported that ASCA reactivity was found 38 mo before clinical diagnosis in 32% of the CD patients studied^[40]; (2) The diagnostic value of serological biomarkers can vary significantly among different ethnic or geographic populations. For example, both ASCA and pANCA were found to be less sensitive in Chinese and Japanese patients^[41,42]. On the other hand, positivity of pANCA was shown to be higher in Mexican-American UC patients: all Mexican-Americans with UC tested had positive pANCA compared to only 40% of Caucasians^[42]. These studies suggest that physicians must factor the patients’ ethnic background when serological biomarkers are applied in the clinical settings.

At least two dozen non-antibody serum biomarkers have also been reported, including, C-reactive protein,

calprotectin, and PMN-elastase, soluble selectins, adhesion molecules, and procalcitonin (PCT)^[4,5,43-45]. However, it is necessary to point out that most of these markers have not been extensively characterized. Many of them are also elevated in a variety of other inflammatory or pathological conditions with a low specificity to IBD. Therefore, their actual clinical value needs to be further investigated or validated.

“NEW” SEROLOGICAL IBD BIOMARKERS

New anti-glycan antibodies: ACCA, ALCA and AMCA

New diagnostic and predicting value: Three new anti-glycan antibodies were first reported as potential novel serological biomarkers in the diagnosis of IBD by Dotan *et al* in 2006 from Glycominds Ltd in Israel^[16,17]. Now, as major components of IBDXTM Panel marketed by Glycominds Ltd (<http://www.ibdx.net/index.html>), this new set of biomarkers contains three anti-glycan antibodies, including anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA), and anti-mannobioside IgG (AMCA) (Table 1). The fourth component in the IBDXTM Panel is gASCA (ASCA IgG), virtually the same as ASCA, which is the first antiglycan IBD serological biomarker identified. Since 2007, several independent studies on these anti-glycan antibodies have been reported, and their clinical utility has been validated by independent laboratories (see below). Glycan, a generic term for all molecules bearing glycosidic bonds, includes mono-, oligo- and ploy-saccharides or carbohydrates^[17]. Glycans are major building blocks of cell surface components and immunogens (erythrocytes, immune cells, and microorganisms) that lead to generation of a variety of anti-glycan antibodies, including IgG, IgA, IgE and IgM, which have been demonstrated in a number of inflammatory and autoimmune diseases^[16]. Mannobioside (AMCA) is a dimer of 1,3 linked mannose, and is a component of mannan from pathogenic fungi and yeast^[12]. Laminaribioside (ALCA) is the building block of laminarin, a polysaccharide of the β -1-3-glucan family and is found in the cell walls of fungi, yeast, and algae^[16,46]. Chitobioside (ACCA) is a component of chitin, found in the insect cuticle and cell walls of infectious pathogens such as bacteria and yeast^[16]. Both β -1-3 glucans and chitin modulates the immune system by binding to receptors on neutrophils, macrophages, and natural killer (NK) cells, thereby stimulating cell proliferation, phagocytosis and cytokine secretion^[47]. The resultant antibody production specifically against chitin and glucan, and their association with CD also suggests the intrinsic modulation of the adaptive immune system. However, the individual diagnostic differences between ALCA, AMCA, and ACCA have not yet been clearly established.

Using GlycoChip glycan array technology^[48,49] and enzyme-linked immunosorbent assay (ELISA), Glycominds Ltd developed the new IBD serological markers (ACCA, ALCA, and AMCA)^[16,17]. The initial study of these 3 new markers, which involved a total of 194 patients with CD, 162 with UC and 142 healthy controls, showed that ACCA, ALCA, and AMCA exhibited the

Table 1 New serological IBD biomarkers

		CD	UC	Control	Ref.
1	ALCA	Approximately 19%-38%	Low (approximately 7%)	Low	16, 17, 18, 50
2	ACCA	Approximately 21%-40%	Low	Low	16, 17, 18, 50
3	AMCA	Approximately 28%	Low	Low	16, 17, 46
4	A Σ MA: Anti- Σ Man3 Anti- Σ Man4	38.60% 22.10% 28.50%	Low	Low	55
5	UBE4A	46.20%	7.10%	3.30%	56
6	CXCL16	Elevated	Elevated		57
7	Apolipoprotein A-IV	Elevated in active CD			58
8	Resistin	Elevated	Elevated		59
9	PF4, MRP8, FIBA and Hpa2				68, 69
10	Cytokines/chemokines & their receptors				

ACCA: Anti-chitobioside IgA; ALCA: Anti-laminaribioside IgG; AMCA: Anti-mannobioside IgG; A Σ MA: Anti- Σ Man3 or Σ Man antibodies; Σ Man3: Synthetic Man α -1,3 Man α -1,2 Man; Σ Man4: synthetic Man α -1,3 Man α -1,2 Man α -1,2 Man; UBE4A: Ubiquitination factor E4A; CXCL16: A transmembrane protein functioning as a chemokine and a scavenger receptor.

highest discriminative capability between CD and UC^[16]. Approximately one third of CD patients are positive for each of the 3 new markers^[16] (Table 1). More significantly, 44% (12/27) of ASCA-negative CD patients were positive for ALCA or ACCA. Therefore, although the prevalence of each of the individual new biomarker is relatively poor (Table 1), together they are a significant complement to ASCA. In patients that were positive with one of the 3 markers, the sensitivity and specificity for diagnosis of CD were 77.4% and 90.6%, respectively. In patients with 2 or 3 of these antibodies, the specificity increased to 99.1%. Higher levels of ALCA and AMCA were significantly associated with small intestinal disease.

Ferrante *et al*^[46] reported a study that involved a larger cohort, including 1225 IBD patients (913 CD, 272 UC, and 40 IC), 200 ethnically matched healthy controls, and 113 patients with non-IBD intestinal inflammation (diverticulitis, infectious colitis, ischemic colitis and pseudomembranous colitis). In this study, IBDXTM Panel (ACCA, ALCA, AMCA, and gASCA) and anti-OmpC were analyzed. 76% of CD patients are positive for at least one of the 5 markers. All antiglycan and anti-OmpC were specific for CD (80.5%-93%). The sensitivity was calculated as: gASCA = 56.4%; ALCA = 17.7%; ACCA = 20.7%; AMCA = 28.1%; and anti-OmpC = 29.1%. Among all 913 CD patients, only 13 (1.4%) were positive for all the 5 CD-associated markers. Fifty percent of CD patients ($n = 435$) who were either sASCA negative/OmpC positive ($n = 93$) or gASCA positive/OmpC negative ($n = 342$), were positive for at least one of the antiglycan markers. Sixty-seven percent of gASCA/OmpC-positive CD patients were also positive for at least one of the other anti-glycan

antibodies. Of 305 sASCA/OmpC-negative CD patients, 7% were ALCA positive, 12% ACCA positive, and 13% SMCA positive. Although addition of ALCA (gASCA/pANCA/ALCA combination) resulted in only minor improvement in differentiating CD from UC compared to the classic gASCA/pANCA combination, it significantly enhanced the accuracy of differentiating IBD from healthy controls and non-IBD intestinal inflammation. Increasing levels of all 5 markers (gASCA, ALCA, ACCA, AMCA, and OmpC) were significantly associated with more complicated disease behavior, including stricture, fistula and need for surgery. However, a recent report by Simondi *et al*^[50] found that, while the level of ASCA appeared to be associated with ileal disease and penetrating/structuring disease, level of ALCA has a similar trend, but did not reach statistic significance ($P = 0.07$ and $P = 0.09$, respectively). This discrepancy may arise from the smaller cohort in Simondi D's study, which involved only 265 subjects (116 CD, 53 UC, 51 healthy controls, and 45 other intestinal diseases).

Similar results on antiglycan antibodies were reported by Papp *et al*^[22] in another study that involved 557 CD patients, 95 UC and 100 healthy controls. 66.2% of CD patients were positive for at least one of the 5 biomarkers tested, including gASCA, ALCA, ACCA, AMCA, and anti-OmpC, all of which were highly specific for CD (79%-100% sensitivity). The sensitivities for each of the 5 markers are gASCA = 50.4%; ALCA = 15.2%; ACCA = 11.3%; AMCA = 11.5%; and anti-OmpC = 31.2% (the 3 new markers, ALCA, ACCA and AMCA were all lower than the results of most other studies^[16,17,46,50]). Overall, increasing levels of these markers were again associated with more complicated disease behavior and incidence of surgery. Among CD patients, gASCA and ALCA were associated with early disease onset (occurring at younger age ($P < 0.0001$ and $P = 0.0012$, respectively, while gASCA was associated with perianal disease ($P < 0.0001$) and azathioprine use ($P = 0.016$). However, no association was found between these serological biomarkers and gender, familial disease, smoking habit and extraintestinal manifestations (EIM). It is interesting to mention that in Simondi D's report^[50], (1) among CD patients, AMCA was found to be significantly higher in women and in smokers than in men ($P = 0.02$) and non-smokers ($P = 0.03$); and (2), CD patients with at least one affected first-degree relative exhibited significantly higher levels of ALCA than those without familial cases (59.8% *vs* 34.7%, $P = 0.0005$), suggestive of a familial association of ALCA.

Association of the antiglycan antibodies with variants of IBD susceptible genes: NOD2/CARD15. Like ASCA^[32,37,38], the newly identified antiglycan antibodies were also found to be associated with single nucleotide polymorphisms (SNPs) of IBD susceptible genes. The first study, reported by Henckaerts *et al* in 2007^[51], examined the influence of mutations in several innate immune receptor genes on the development of anti-glycan and anti-OmpC antibodies in IBD, including NOD2/CARD15, NOD1/CARD4, TUCAN/CARDI-

Table 2 A gene dosage effect: % positivity of antiglycan antibodies in CD patients carrying zero, one and two NOD2/CARD15 variants

	CD patients with 0 NOD2/CARD15 variant	CD patients with 1 NOD2/CARD15 variant	CD patients with 2 NOD2/CARD15 variants	P	Ref.
gASCA	51.50	64.20	72.30	< 0.0001	51
	41.60	64.60	67.50	< 0.0001	22
ALCA	34.90	42.10	46.70	< 0.04	51
AMCA	9.80	14.10	30	< 0.001	22
Any antiglycan	52.40	69.70	80	< 0.0001	22

NAL/CARD8, Toll-like receptor (TLR) 4, TLR2, TLR1 and TLR6. The study involved 1163 unrelated IBD patients (874 CD, 259 UC, and 30 IC) and 312 healthy controls. CD patients with at least one NOD2/CARD15 variant (1) were more frequently gASCA or ALCA positive than those with no mutation (gASCA: 66.1% *vs* 51.5%, $P < 0.0001$; ALCA: 43.3% *vs* 34.9%, $P = 0.018$); and (2) had higher gASCA titers (85.7 *vs* 51.8 ELISA units, $P < 0.0001$). This association was independent of ileal involvement. More interestingly, a gene dosage effect was observed when positivities of antiglycan antibodies in CD patients carrying 0, 1 and 2 NOD2/CARD15 variants were compared. Anti-glycan positivity became more frequent as the number of NOD2/CARD15 mutations increased (Table 2).

A remarkably similar gene dosage effect on gASCA was observed by Papp *et al*^[22] in 2008 (Table 2) as compared to Henckaerts L's study^[51]. They analyzed the association of antiglycan with NOD2/CARD15 variants from studying 557 CD patients, 95 UC and 100 healthy controls. When comparing CD patients with NOD2/CARD15 variants to those with wild type alleles, positivity of gASCA is 65.2% *vs* 41.8% ($P < 0.0001$), AMCA is 18.8% *vs* 9.7% ($P = 0.009$), and any antiglycan is 72.5% *vs* 52.5% ($P < 0.0001$). In addition, Papp M's report also showed the gene dosage effect on AMCA and any antiglycan antibodies (Table 2).

NOD1/CARD4, TLR2, and TLR4: In Henckaerts L's report^[51], CD patients carrying one GG-indel allele in NOD1/CARD4 had a higher prevalence of gASCA than those with wild type allele (63.8% *vs* 55.2%, $P = 0.014$). Gene dosage effect of NOD1/CARD4 was also evident as the number of mutant alleles increased, but did not reach statistical significance. Interestingly, CD patients with at least one mutation TLR4 (D299G) had a lower prevalence of ACCA compared with TLR4 wild type (D299A) (23.4% *vs* 35%, $P = 0.013$), and a lower ACCA titer (39 *vs* 49 EU, $P = 0.05$). An inverse gene dosage effect of TLR4 was observed: the prevalence of ACCA is 34.9%, 21.1% and 9.1% in CD patients with 0, 1 and 2 NOD1/CARD4 variants, respectively. A similar inverse gene dosage effect was also seen for anti-OmpC prevalence gene in relation to the number of TLR2 mutations. Of note, other reports did not find any

significant association of TLR4 (25) or NOD1/CARD4 (variant E266K)^[52] with the positivity of any antiglycan antibodies.

DEFB1: Lakatos *et al.*^[52] recently reported an association of the antiglycan antibodies with specific variants of β -defensin-1 (DEFB1). In this study of a sex-matched cohort of 276 CD patients and 100 healthy controls, four IBD susceptible genes were tested, including NOD2/CARD15, NOD1/CARD4, DLG5, and DEFB1. Two DEFB1 variants, G20A and C44G, were found to be inversely associated with the positivity of antiglycan antibodies. For example, 29.6% of DEFB1 20A carriers were antiglycan positive compared to 46% positivity in non-carriers ($P < 0.038$). However, no association of antiglycan positivity was found with either DEFB1 G52A variant or DLG5 (R30Q), although both were shown to be associated with increased risk for CD^[53,54]. Furthermore, in contrast to other reports^[32,51], no gene dosage effect was observed on any of the antiglycan antibodies.

Anti-synthetic mannoside antibodies: Evidence for the existence of a new subset of antiglycan antibodies

Based on the chemical structure of mannose epitopes for ASCA, the antibodies against mannose residues [Man α -1,3 (Man α -1,2 Man) 1-2] that are the most widely recognized biomarkers for CD, Vandewalle-El Khoury *et al.* recently^[55] chemically synthesized (Σ) two major oligomannose epitopes, Man α -1,3 Man α -1,2 Man (Σ Man3) and Man α -1,3 Man α -1,2 Man α -1,2 Man (Σ Man4). Their goals were to test the immuno-reactivity of the Σ Man3 and Σ Man4 with specific serum antibodies (termed “A Σ MA” for anti-synthetic mannoside antibodies) and compared A Σ MA with ASCA for their values as serological biomarkers for CD. An impressively large cohort was used in the study, including a total of 1365 subjects (772 CD, 261 UC, 43 IC and 289 controls).

Overall, while the specificity of A Σ MA for CD was quite similar to that of ASCA (89% *vs* 93%), the specificity was lower (38% *vs* 55%). 16% of CD patients were positive for both ASCA and Σ Man3, and 24% positive for Σ Man4. Interestingly, 11% of ASCA-negative CD patients were A Σ MA positive (5% for Σ Man3, 4% for Σ Man4, and 2% for both). Together 24% of CD patients who were negative for ASCA and/or other CD-associated serological biomarkers were positive for A Σ MA, suggesting a previously unrecognized new subset of anti-mannose antibodies are present in patients with CD. Therefore, it is conceivable that a combination of A Σ MA with ASCA, ACCA, ALCA and AMCA would significantly increase the diagnostic value of the existing panel of antiglycan biomarkers.

A Σ MA was analyzed for its predictive value for the evolution of IC patients as well as the involvement of disease location of CD (small bowel *vs* colon). Twenty IC patients (out of a total of 43) evolved to a final diagnosis of CD ($n = 11$; UC = 7; UC-like CD = 2). Among 11 patients with final diagnosis as CD, one was ASCA-positive/A Σ MA-negative, two were ASCA-positive/A

Σ MA-positive, and three were ASCA-negative/A Σ MA-positive. None of the UC patients were A Σ MA-positive. Therefore, A Σ MA was more sensitive (45% *vs* 27%) and more specific (100% *vs* 71%) than ASCA for predicting evolution of IC toward CD. As for the predictive value of disease location, although positivity of A Σ MA had no association with any particular disease phenotype, among the ASCA-negative CD patients, A Σ MA positivity was significantly associated with colonic involvement. This indicates that A Σ MA may provide new diagnostic value to colonic CD, for which ASCA is less frequently detected.

Other serum/plasma biomarkers

Ubiquitination factor E4A (UBE4A): By screening a phage library from normal terminal ileum with sera from patients with CD, Sakiyama *et al.*^[56] identified a strongly immunoreactive cDNA clone encoding the C-terminal subunit of the UBE4A, a U-box-type ubiquitin-protein ligase. To investigate the specificity of the serum anti-UBE4A autoantibodies in CD patients *vs* UC patients *vs* healthy controls, a GST-C-terminal UBE4A fusion protein was made, and used to test the immunoreactivity of sera from 39 patients with CD, 28 with UC, and 60 healthy controls. The prevalence of anti-UBE4A IgG was significantly higher in CD than that in UC or healthy controls (46.2% *vs* 7.1% *vs* 3.3%, respectively; $P < 0.0006$) (Table 1). The levels of anti-UBE4A IgG were correlated well with the disease activity ($P < 0.0001$). More interestingly, higher level of anti-UBE4A IgG was associated with complicated disease behavior (stricturing and penetrating) ($P = 0.0028$), and patients positive with anti-UBE4A IgG were more likely to undergo surgery ($P = 0.0013$). Although UBE4A expression was low in the cytoplasm of enterocytes and goblet cells, immunohistological analysis showed that UBE4A expression was highly elevated only in enteroendocrine cells of ileal mucosa from CD patients, but not in normal subjects. It was speculated that production of anti-UBE4A autoantibodies might be a result of increased expression of UBE4A in the inflamed ileal mucosa. The exact role of UBE4A elevation and production of anti-UBE4A autoantibodies in the pathogenesis of CD remains to be determined.

CXCL16, apolipoprotein A-IV, and resistin: These newly reported serum/plasma IBD biomarkers were reported by the same working group in Germany^[57-59]. It appears that the same cohort of study subjects was used for all three studies, or at least cohorts might have some overlapping subjects. Further validation study of these markers by other independent laboratories is necessary to evaluate their usefulness.

CXCL16 (Table 1), an intriguing transmembrane protein composed of an extracellular chemokine domain fused with a mucin stalk that extends through cell surface, functions as a chemokine and a scavenger receptor and has been implicated in various inflammatory diseases. It becomes soluble after being cleaved by metalloproteinase ADAM 10 and exerts chemokine functions^[60].

Lehrke *et al*^[57] reported that CXCL16 could potentially be a surrogate IBD biomarker after having examined the serum levels of soluble CXCL16 in a cohort of 239 patients with CD, 114 UC, and 144 healthy controls. Soluble CXCL16 levels were found to be the highest in CD patients ($P < 0.001$) compared with UC and healthy controls. UC patients had a relatively modest, but significant elevation of CXCL16 compared with healthy controls ($P < 0.001$). No significant difference was seen between active and inactive state of CD or UC.

Apolipoprotein A-IV (Table 1), a structural component of intestine derived triacylglycerol-rich chylomicron particles with anti-oxidant, anti-atherogenic, and anti-inflammatory properties, has been recently shown to inhibit DSS-induced mouse colitis^[61]. For this reason, Broedl *et al*^[58] tested if plasma level of apolipoprotein A-IV was associated with IBD, and found that it was inversely associated with disease activity and CRP levels in patients with CD (but not UC) ($P < 0.005$). However, since the actual difference in the level of apolipoprotein A-IV between active and inactive CD is quite small, the clinical value of this marker remains to be determined.

Resistin (Table 1), also known as adipocyte secreted factor or FIZZ-3, is a peptide hormone that is associated with multiple inflammatory conditions^[62]. Studies by Konrad *et al*^[59] showed that patients with both CD and UC had significantly higher plasma levels of resistin compared to healthy controls ($P < 0.0001$). The levels of resistin in both CD and UC were significantly associated with white blood cell count ($P < 0.0001$), CRP ($P < 0.0001$), and disease activity ($P < 0.0001$).

Application of proteomic and infrared spectroscopic technologies in serological IBD biomarker profiling

The rapid development of proteomic technologies recently has revolutionized the way and capacity by which biomarker discovery is performed^[63-67]. Current proteomic methodologies include three sub-categories: mass spectrometry (MS)-based technologies, array-based technologies and imaging MS (see review^[66]). Blood is the most explored source for disease biomarkers by proteomics^[63-65,67]. Proteomics of IBD was recently reviewed^[80].

SELDI-TOF-MS and MALDI-TOF-MS: Unlike the quests for biomarkers of other major diseases (such as cancers), application of proteomic technologies in IBD biomarker discovery is only in its infancy. So far (as of June 17, 2008) only three full original MS-based reports are available, of which, two used SELDI-TOF-MS by Merville/Louis groups^[68,69] and one used MALDI-TOF-MS by Roda's group^[70]. Using SELDI-TOF-MS (Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometer), Meuwis *et al*^[68] analyzed protein profiles of 120 serum samples collected from a cohort of 30 CD, 30 UC, 30 inflammatory controls and 30 healthy controls. Multivariate analysis generated models that could classify samples with minimum 80% sensitivity and specificity in discriminating groups of patients. Four peptides were identified from potential peaks that could best discriminate the four groups, lead-

ing to the identification of 4 serum biomarkers, including platelet aggregation factor 4 (PF4), MRP8 (S100A8), FIBA (a peptide released during clotting from fibrinogen precursor) and Hpa2 (haptoglobin $\alpha 2$) (Table 1). The diagnostic value of these markers remains to be further examined. Using similar approaches, the same group piloted a study of sera from 20 CD patients who showed either response or no response to infliximab^[69]. PF4 was again identified as a potential marker. The intensity level of SELDI peak in which PF4 was identified was inversely associated with infliximab non-responders. Unfortunately, such association could not be confirmed by ELISA measurement of PF4. Also, PF4 did not exhibit a significant correlation with other disease markers (sCD40L, IL-6, and CRP) or CDAI, casting doubt that PF4 probably would have any clinical diagnostic value.

The third MS-based profiling of serum IBD markers was reported by Nanni *et al*^[70] using MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization Time of Flight-Mass Spectrometer). The study, which involved a small cohort (15 CD, 26 UC and 22 healthy controls), found the reversed-phase extraction and selection of 20 m/z value gave the best overall predictive value (96.9%). In another study, reported at Digestive Disease Week (DDW) 2008, Subramanian *et al*^[71] analyzed sera from a cohort of 62 UC and 48 CD by SELDI-TOF MS. Bio-statistical analysis identified 12 discriminative peaks, with specificity and sensitivity approximately 95% (compared to 80.9% of the sensitivity of ASCA for CD and 64.5% of pANCA for UC). Four serum proteins were identified as inter alpha trypsin inhibitor 4, apolipoprotein C1, platelet activated factor 4 variant, which are expected to be further analyzed for their clinical utility.

Protein and antibody arrays/chips: Compared with other proteomic approaches, protein/antibody array (or chip) array technologies offer the advantages of being highly specific with high-throughput nature and capacity. The application of these technologies in IBD biomarkers discovery has just begun, and thus, the data are limited. Since 2006, our laboratory began using proteins that were robotically spotted on array slides as bait to screen serum IBD biomarkers^[72]. Since the currently known major serological IBD biomarkers or antibodies are against microbes or protein of human origin, including ASCA, ANCA, OmpC, Cbir, and the new antiglycan antibodies, we hypothesized that disease-specific antibodies to microbial or to human protein (autoantibodies) are present in patients' sera (manuscript in preparation). We found to our surprise that human sera contain antibodies immuno-reactive to hundreds of proteins from *E. coli*, yeast, and even humans. The numbers and immunoreactivity of these antibodies vary greatly among IBD patients and even among healthy individuals. From our experiences, when protein arrays are used, the serum quality must be high, the screening process must be standardized, and sufficient number of subjects (at least approximately 30 per comparing group) should be included. This results in a high cost of experiments due to the expense of the commercial protein- or antibody arrays (unless made using assembled robotic ar-

rayers). One presentation by Vermeulen *et al.*^[73] at DDW 2008 reported a study of using commercial human protein arrays to profile serum IBD biomarkers from a very small cohort of subjects (10 UC, 15 CD and 5 healthy controls). They found that 75 proteins reacted more strongly with sera from IBD than those from healthy controls, while reactivity of another 88 proteins was just opposite. One identified antigen, described as an autoantigen in IBD was pleckstrin homology-like domain, family A, member 1 (Phla1). Validation experiment using a larger cohort of subjects found that approximately 46% of IBD [UC: 42.8% (27 of 63); CD: 50.0% (33 of 66)] were positive for anti-Phla1 antibodies, compared to 28.7% healthy controls (19 of 66) and 33.3% non-IBD gastrointestinal controls (22 of 66). Therefore, the discriminative power of this anti-Phla1 for CD *vs* UC or IBD *vs* controls is poor.

So far, antibody arrays were used in only one report by Kader *et al.*^[74] for identifying IBD serum biomarkers. In this study, antibody arrays containing 78 cytokines, growth factors, and soluble receptors were used to screen 65 patients with CD and 23 with UC. Univariate analysis found that the levels 4 cytokines (PLGF, IL-7, IL-12p40, and TGF- β 1) were significantly elevated in patients with clinical remission compared to active disease ($P < 0.01$). However, only the difference in IL12p40 reached statistical significance ($P < 0.02$).

Fourier transform near-infrared spectroscopy: Haas *et al.*^[75] reported at DDW 2008 a new application of Fourier Transform Near-Infrared Spectroscopy (FT-NIR) in serum biomarker profiling. Specific spectra or fingerprints of serum samples from 139 patients with CD and 120 with UC were obtained by FT-NIR, and analyzed by Artificial Neural Networks (ANN) and cluster analyses. ANN-analysis showed that the sensitivity, specificity and accuracy for IBD *vs* healthy controls were 94.5%, 99.1%, and 96.7%, respectively, compared to cluster analysis (71.8%, 66.6%, and 70.1%). Cluster analysis correctly identified 80% of UC and 61.5% of CD, while ANN-analysis was 69.8% and 91.8%, respectively. This proof-of-concept study suggests a potential usefulness of this technology in identifying serum “fingerprints” as serological biomarkers for IBD diagnostics.

Serum cytokines/chemokines and their receptors

Serum cytokines and their soluble forms of receptors have been extensively studied, both as markers for IBD diagnosis and as molecules for IBD pathogenesis, as well as IBD therapeutic targets^[4,76-78]. A long list of cytokines/chemokines and their receptors have been shown to up-regulated in active and even inactive IBD, including IL-1 α , IL-1 β , IL-1R (R: receptor), IL-1Ra (Ra: receptor antagonist), IL-2, sIL-2R (s: soluble), IL-6, IL-6R, IL-7, IL-8, IL-10, IL-12, IL-15, IL-16, IL-17, IL-18R, IL-27, IFN- α , IFN- β , TGF- β , TNF- α , TNF- α R, as well as most chemokines. However, the diagnostic value of cytokines/chemokines has been limited, at least in part due to studies based on the analysis of individual or only a few selected cytokine/chemokines. The ideal situation would be to profile a large number of serum

cytokines/chemokines from well characterized cohorts. An example of this is the utility of a more robust and high-throughput multiplex sandwich ELISA (which allows simultaneous analysis of up to 100 analytes), in conjunction with biostatistical analysis tools [such as that of discriminant functional analysis (DFA) and multidimensional scaling (MDS)], as we and others have described^[79-83]. As a proof-of-concept in the context of IBD, we recently analyzed 16 serum cytokines by multiplex ELISA from a cohort of 64 mice with or without induced experimental colitis^[84]. Distinctive disease-specific cytokine profiles were identified with significant correlations to disease activity and duration of disease. Our data showed that CD-like TNBS colitis exhibits heightened Th1-Th17 response (increased IL-12 and IL-17) as the disease becomes chronic. In contrast, UC-like DSS colitis switches from a Th1-Th17-mediated acute inflammation (increased TNF α , IL6, IL-17 and KC) to a predominant Th2-biased chronic inflammatory response (increase in IL-4 and IL-10 with concomitant decrease in TNF α , IL6, IL-17 and KC). DFA identified 5 discriminatory cytokine biomarkers (IL-6, 12, 4, 17, INF- γ) that can sufficiently distinguish healthy controls from diseases, and one disease type from another^[84]. A pilot study profile of 17 human cytokines/chemokines from a small cohort of 33 IBD patients (19 CD and 14 UC) with 33 matched healthy controls using multiplex ELISA also identified patterns of cytokines/chemokines that were correlated with disease phenotypes (CD *vs* UC) and severity^[82]. A similar human cytokine profiling study using 24 cytokines/chemokines from a larger cohort of 400 IBD patients (with various levels of disease severity) is near completion. It will be interesting to see if the cytokine profiles can: (1) enable disease subtype stratification; (2) correlate with disease severity; and (3) if the profiles that were identified in murine experimental colitis would be similar to those of human IBD.

CONCLUSION

It is evident that while current serological IBD biomarkers are useful, their clinical utility has been limited. New technologies, such as those described in this highlight, demonstrate the significant potential for identifying previously unrecognized IBD biomarkers. Future direction is predicted to be, in addition to the continuation of ongoing efforts in developing novel biomarkers using conventional and new technologies, the integration of multiple biomarkers with extensive bioinformatics analysis/modeling. This will be the key to eventually developing specific “endpoint-oriented” serological biomarker kits. These may include, but not be limited to, individual biomarker sets that are specific for one or more of the following: (1) differentiating CD *vs* UC *vs* normal *vs* other non-IBD gut diseases that share some similar clinical presentations (such as abdominal pain and diarrhea in infectious colitis or IBS); (2) predicting IBD risk (before disease onset; subclinical biomarkers) and disease course (risk of complication and surgery); (3) predicting therapeutic efficacy even before initiation of specific

medication(s); and (4) monitoring therapeutic efficacy and predicting relapse. One can envision that such kits will rely on “integrated algorithms”, rather than absolute differences, to enhance the accuracy of diagnosis and/or prognosis of IBD.

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Helminth infections and intestinal inflammation

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Abstract

Evidence from epidemiological studies indicates an inverse correlation between the incidence of certain immune-mediated diseases, including inflammatory bowel diseases (IBD), and exposure to helminths. Helminth parasites are the classic inducers of Th2 responses. The Th2-polarized T cell response driven by helminth infection has been linked to the attenuation of some damaging Th1 driven inflammatory responses, preventing some Th1-mediated autoimmune diseases in the host, including experimentally induced colitis. Helminth parasites (the porcine whipworm, *Trichuris suis*) have been tested for treating IBD patients, resulting in clinical amelioration of the disease. As a result, there is a great deal of interest in the research community in exploring the therapeutic use of helminth parasites for the control of immune-mediated diseases, including IBD. However, recent studies have provided evidence indicating the exacerbating effects of helminths on bacterial as well as non-infectious colitis in animal models. Therefore, a better understanding of mechanisms by which helminths modulate host immune responses in the gut may reveal novel, more effective and safer approaches to helminth-based therapy of IBD.

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HELMINTH INFECTIONS

Helminths are divided into two major phyla, and all members of each phylum have distinct structural features that separate them from the others. The nematodes (also known as roundworms) include the major intestinal worms and the filarial worms that cause lymphatic filariasis (LF), whereas the platyhelminths include the trematodes (flukes), such as the schistosomes, and the cestodes (tapeworms). Humans and helminths have co-existed throughout our evolution. The history of helminthiasis can be tracked back to the earliest record of human beings^[1]. Although effective preventive and therapeutic measures have been developed for most parasitic worms, helminth infections are still very common in the developing world today. It has been estimated that one billion people worldwide are infected with one or more helminths. Most of the victims live in regions of sub-Saharan Africa, Asia, and Latin America^[2-3]. The most common forms of helminthiasis are infections caused by intestinal helminths, ascariasis, trichuriasis and hookworm, followed by schistosomiasis and LF^[4]. Clinical features of helminthiasis vary a lot depending on the helminth species, intensity of infection, and host age. *Taenia solium* can cause neurocysticercosis with mass lesions in brain. Ingested eggs of *Echinococcus granulosus* will lead to cysts in the liver and cause life-threatening anaphylaxis if antigens are released from the cysts. Chronic infection with *Schistosoma* causes granulomas, fibrosis, and inflammation of the spleen and liver. Hookworm and schistosomiasis can infect pregnant women, cause neonatal prematurity and increased maternal morbidity and mortality^[5]. Children at school-age or younger tend to harbor the greatest numbers of intestinal worms and schistosomes compared with any other age group. As a

result, the young patients suffer from growth retardation and diminished physical fitness, as well as memory and cognition impairment^[6]. Most helminth infections, if left untreated, result in multi-year, chronic inflammatory disorders that will eventually cause disability. The chronic, disabling and disfiguring consequences of helminth infections, together with their high prevalence, make them a global problem of significant medical, educational, and economic impact.

HELMINTH INFECTION INDUCES TH2 AND T REG DEVELOPMENT

Since parasitic worms constitute a very heterogeneous collection of organisms, a multitude of mechanisms may be expected when they stimulate and modulate host immune responses. However, examination of the immunology of helminth infections reveals a number of characteristics that are generally conserved across all species. Our current understanding of the immune response to helminth infections has largely come from the study of well-defined laboratory models of infection in rodents. Protective immunity to helminths depends on T lymphocytes. It is now well established that the CD4⁺ subset of T cells plays a major role in the generation of the host protective response that expels the worms, and that CD4⁺ T cells regulate many of the inflammatory and immune parameters that accompany expulsion of the parasites from the gut. Based on cytokine production profiles, CD4⁺ T cells can be divided into distinct functional sub-populations, Th1, Th2 and Th17. Th1 cells produce cytokines such as IFN- γ , IL-12 and IL-2 that are involved in controlling of intracellular pathogens. These cells also contribute to inflammatory responses. Recently, a new lineage of Th cells, that selectively produce the cytokine IL-17 (Th17) has been identified, and those cells are thought to be key regulators of inflammation^[7,8]. It has also been reported that IL-17 is required for the optimal induction of Th1 and Th2 immune response, although the mechanism has not yet been clarified^[9]. IL-17 is a proinflammatory cytokine, which is produced predominately by activated T cells. This cytokine can enhance T cell priming and induces the activation of many cell types including fibroblasts, macrophages and epithelial cells, resulting in the production of multiple proinflammatory mediators by these cells, including IL-1, IL-6, TNF- α and chemokines^[8,10]. In the majority of cases, the immune responses of the hosts to worm infections are strikingly dominated by Th2-like T-helper cell responses with a significant production of IL-4, IL-5, IL-10, IL-13, IL-25, and IL-31^[7,8,11,12]. Th2 cells contribute to B cell activation and antibody production, eosinophil differentiation and recruitment. Therefore, parasitic worm infection is often associated with high levels of IgE, IgG1 and IgG4, and robust eosinophil and mast cell responses. Th2 immune responses to helminth infections can prevent the survival of invading parasites during a homologous secondary infection^[9], expel adult parasites from the gut^[10], and

allow host survival when the immune responses are not able to clear the parasites. These responses are the basic host protective mechanisms against worms and are highly conserved through evolution. However, Th2 immune responses to helminth infections may also cause host pathology and organ damage. For example, Th2 immunity can mediate pathological fibrotic responses in chronic parasitic infections^[13]. This can be understood as the detrimental effects of host immunity to helminths. Cross-regulatory suppression of the Th1 responses by a strong Th2 response has been considered to play a role in modulating diseases that are characterized by a Th1 response. Multiple studies^[14-16] have revealed that helminth-induced Th2 responses can attenuate damaging Th1-driven inflammatory responses in the host. However, suppression of Th1 response may also lead to the impairment of host protective Th1 immunity against concurrent infections caused by bacterial, viral and parasitic pathogens, exacerbating the diseases they induce.

In addition to stimulating a vigorous Th2 response, helminth infections are also capable of inducing suppressive T cell populations known as regulatory T cells (Tregs)^[17], which may help control morbidity and dampen resistance to re-infection through their potent immune regulatory mechanisms. Several types of Treg cells have been described (natural Tregs, Tr1 and Th3). These regulatory cells constitute 5% to 10% of peripheral CD4⁺ T cells in naive mice and humans and suppress several potentially pathogenic responses *in vivo*, particularly T cell responses directed to self-antigens. These cells express markers such as Foxp3, CD25, CTLA-4 and GITR, and often secrete IL-10 and/or TGF- β ^[18-20]. Tregs have been shown to play an important role in regulating immune responses and maintaining homeostasis under various disease conditions including autoimmune disease, inflammation, cancer, and microbial infections. Treg regulate immunity through both cytokine-dependent and independent mechanisms^[21]. Th3 cells make TGF- β , which inhibits development of both Th1 and Th2 cells. Tr1 cells regulate immunity through IL-10-dependent mechanisms, which inhibit both Th1 and Th2 responses^[22-24]. A recent study has provided a link between the well-established immunoregulatory capacity of Th2 cells and Tregs showing that the IL-4Ra binding cytokines, IL-4 and IL-13 induce FOXP3 expressing Treg from CD4⁺CD25⁺ precursors^[25].

Existing data indicate that helminth infections induce the development of Th2 and/or Treg responses. A polarized Th2 type immune response as well as up-regulated regulatory T cell activity induced by helminths may have a significant impact on the host's ability to cope with concurrent or subsequent viral^[26,27], bacterial and parasitic infections^[28], by suppressing host protective Th1 responses to microbial pathogens. Consistent with the hygiene hypothesis, helminth-induced responses may also underlie the observed reverse associations between helminthiasis and asthma, allergy, IBD, and

other autoimmune diseases^[29-31]. A better understanding of immune modulation by helminth infection, therefore, can have significant practical implications for the prevention and treatment of immune-mediated as well as microbial disease.

HELMINTHS AFFECT HOST RESPONSES TO OTHER ANTIGENS AND PATHOGENS

The major importance of helminth infections includes not only the direct pathogenic effect of the worms as described above, but also the modulation of the host immune system, which may alter the response to other pathogens and antigens and cause additional immunopathology. The distribution of several pathogenic helminth infections coincides geographically with many devastating microbial diseases, such as HBV, HCV, HIV/AIDS^[26,27,32-34] and malaria^[35-37]. Coinfection with helminths increases the transmission of and susceptibility to these infections, and also increases the severity of the associated diseases^[27,33-38]. Recently we have demonstrated that an intestinal nematode parasite, *Heligmosomoides polygyrus* (*H. polygyrus*) infection impairs host Th1 defense against enteric bacterial infection and exacerbates *Citrobacter*-mediated intestinal injury^[28,39]. This observation is keeping with a report showing that infection with the helminth *Fasciola hepatica* reduced the protective Th1 response to coinfecting *Bordetella pertussis* and exacerbated the bacterial infection^[40]. Likewise, infection with *Schistosoma mansoni* downregulated antigen-specific Th1 cytokines and cytotoxic-T-lymphocyte responses, resulting in a delay in vaccinia virus clearance^[41]. Moreover, it has been shown that a combined infection with *Trichuris suis* and *Campylobacter jejuni* in immunologically naive, germfree piglets, resulted in an enhanced invasion of the colon by *C. jejuni*, leading to the development of more severe pathology^[42]. These observations provide strong evidence to demonstrate that helminth infection can dampen Th1 reactions to other infections and cause impaired immune responses to concurrent viral, bacterial, and parasite infections, as well as to vaccination. The Th2-inducing helminth infection has also been shown to inhibit the development of CD8+ T cell responses^[43].

HELMINTHS MODULATE INFLAMMATORY DISORDERS OF THE HOST

It has been observed that there is an increased incidence of autoimmune disorders and allergic diseases in the developed world. This phenomenon is thought to be the consequence of eradication of major infectious diseases, including helminth infections in this part of the world, a theory termed the hygiene hypothesis. The inverse correlation observed between helminth infection and certain immune-mediated diseases has led to the suggestion that lack of helminth infections favors the induction of Th1 responses, which may, in turn, result

in the clinical appearance of gastrointestinal diseases, including inflammatory bowel diseases (IBD)^[44-46]. IBD, including Crohn's disease and ulcerative colitis are chronic immune diseases of the gastrointestinal tract with unknown etiology. The current hypothesis indicates that IBD results from an uncontrolled immune response to the normal gut flora^[47]. Genetic factors and environmental factors both contribute to the damaging mucosal immune response^[48]. The hygiene hypothesis suggests that microbes and worms are important for shaping and tuning the development and function of our immune system^[49]. The growing body of epidemiological and experimental data strongly suggest that a reduction in helminth infection is linked to rising rates of autoimmunity and atopy.

The initial work by Elliott *et al.*^[50] showing a protective response of *Schistosoma mansoni* infection on trinitrobenzene sulphonic acid (TNBS)-induced colitis in mice, a chemically induced Th1-type colitis used as an experimental model of human IBD, have led to several animal studies determining the role of helminth infections in different IBD models. In 2001, Reardon *et al.*^[51] showed that infection of mice with the tapeworm *Hymenolepis diminuta* ameliorated dextran sodium sulphate (DSS)-induced colitis. Khan *et al.*^[52] showed that mice that were infected with the nematode, *Trichinella spiralis* are protected from colitis induced by intrarectal challenge with dinitrobenzene sulphate (DNBS). Furthermore, schistosome eggs also provide a protective effect on TNBS-induced colitis in mice^[53] and infection with *H. polygyrus* or *T. muris* can prevent or reverse the chronic spontaneous Th1-type colitis in IL-10 deficient mice^[46]. A protective effect of infection with *Schistosoma mansoni* on TNBS-colitis in rats was also reported^[54]. These observations provide evidence to suggest that helminth parasites (nematode, cestode and trematode) can ameliorate chemically induced colitis in different models. In line with these observations, the Th2 polarized T-cell response driven by helminth infection has also been linked to the amelioration of some Th1-mediated diseases that develop concurrently, such as *Helicobacter*-induced gastritis^[14].

Evidence not only suggests that helminths can attenuate experimentally induced IBD in animal models^[42,53,54], but also the pioneering work of Weinstock *et al.* shows promise using *Trichiuris suis*, a pig helminth, to treat Crohn's patients^[16]. Therapeutic potential has also been indicated in CD patients who were infected with the human hookworm *Necator americanus*^[55]. The effects of helminth on host intestinal Th1 function could be mediated through several mechanisms, including helminth-induced Th2 response and induction of Tregs. We have shown that infection with the intestinal helminth *H. polygyrus* results in an upregulation of colonic IL-4 expression^[28]. Recently Setiawan *et al.*^[56] have provided evidence to show that *H. polygyrus* infection promotes Th2 cytokine responses (IL-4, IL-5 and IL-13) of colonic lamina propria mononuclear cells. Helminths also induce production of powerful immune modulatory

molecules like IL-10 and TGF- β ^[57] that can affect both Th1 and Th2 function.

Unfortunately, the anticolitic effect evoked in response to helminths in mice does not extend to all models of colonic inflammation. In a recent study, the ability of the rat tapeworm *Hymenolepis diminuta* to affect the course of oxazolone-induced colitis was determined. A detrimental effect of helminth infection on colitis was detected, as evidenced by the results showing that *H. diminuta* caused a significant exacerbation of oxazolone-induced colitis^[58].

Considering the profound and widespread immune activation and dysregulation induced by helminth parasites, the overlapping geographic distributions of helminth and bacterial infections, and the potential to modulate bacteria-associated intestinal inflammation, we have established a co-infection model system to analyze the effect of an intestinal helminth parasite on a concurrent enteric bacterial infection and bacteria-mediated intestinal inflammation^[28]. This model involves two murine enteric infectious agents that induce distinct Th-responses: (1) the Th2-inducing helminth *H. polygyrus*, and (2) the bacterial pathogen, *C. rodentium*, which selectively stimulates a Th1-type immune response^[59,60]. *C. rodentium* is a mouse pathogen that colonizes the distal colon and causes pathological changes that are similar to those seen in many mouse models of colitis, including transmissible colonic hyperplasia, goblet cell depletion, and mucosal erosion. *C. rodentium* has been used as a model for studying host responses to human pathogens that employ attaching and effacing (A/E) lesion formation for epithelial colonization, such as EPEC, the most important causative agent of severe infantile diarrhea^[59,61-64]. We found that mice co-infected with *H. polygyrus* developed a more severe *C. rodentium*-associated colitis. The exacerbation of bacterial colitis induced by helminth co-infection is STAT6 dependent. These results provide evidence to indicate the possibility that helminth infection may have adverse effects on intestinal inflammation (bacteria-mediated as well as inducible colitis). In addition, it has been also reported that *T. suis* ova treatment resulted in infection in the gastrointestinal tract of a pediatric Crohn's patient^[65]. These observed deleterious consequences of helminth parasites in intestinal inflammatory responses, therefore, provide a cautionary note for the therapeutic use of helminths in certain forms of IBD. These observations also highlight the need for a more comprehensive understanding of the mechanisms by which helminths modulate host's responses to enteric bacteria and bacteria-associated as well as immune-mediated intestinal inflammation.

IMPACT OF HELMINTHS ON INNATE IMMUNE OF THE HOST

Innate immune cells such as DCs and macrophages are fundamental to directing immune responses along either a tolerating or activating pathway. As master

manipulators of the host immune system, helminths have evolved strategies targeting these cells. To effectively control infection, appropriately developed and functionally polarized subsets of Th effector cells are required. DCs, the most competent antigen presenting cells, play an important role in the initiation of both innate and adaptive immune responses to a wide variety of pathogens and Ags, as well as in tolerance^[66-69]. DCs can be activated by microbial products through their high-level expression of evolutionarily conserved pattern recognition receptors, such as Toll-like receptors (TLRs)^[70,71]. The signals derived from various types of microbial components, such as those from helminth and bacteria, are translated by the DCs into a stimulus for T cells, leading to a productive T cell response. The T cell stimuli derived from DCs include MHC II-peptide (signal 1), the expression or absence of costimulatory molecules (signal 2), and expression or absence of polarizing cytokines (signal 3)^[72,73]. Microbial antigens can activate DCs through ligation of TLRs, leading to the up-regulation of functional surface molecules, and the release of cytokines, such as IL-12, IL-18 and IL-27, resulting in the induction of Th1 immunity^[70,71,74]. Although it is less clear, there is also evidence indicating that DC function is modulated during helminth infection^[75]. Different helminth-derived molecules have been suggested to be able to induce DC activation, and subsequent Th2 and/or T reg responses. Studies have indicated that two *Schistosoma* egg-derived components (the glycolipid lysophosphatidylserine and the carbohydrate determinant lacto-N-fucopentaose III) can activate TLR4 and TLR2, respectively, in myeloid DCs^[76]. Live schistosoma eggs can activate DCs through TLR2 and TLR3^[77]. Parasite secreted compounds like ES-62 from filarial nematodes induce the development of DCs and are capable of driving a Th2 response, modulating cytokine production (IL-12 and TNF- α) in macrophages and DCs *via* the TLR4 pathway^[78]. A recent report suggests that helminth infection may alter TLR 4 expression in mucosal T cells, and these T cells when stimulated with LPS produce TGF- β ^[79]. Additionally, murine bone marrow-derived DCs pulsed with the helminth excretory/secretory antigen can, on transfer to naive recipients, prime mice for Th2 responsiveness^[80]. Other studies also indicate that the soluble fractions from *S. mansoni* eggs may alter TLR ligand-induced activation of DCs^[81]. These results clearly indicate the possibility that helminth infection may modulate TLR expression of DCs and responsiveness of DCs to TLR ligands.

In addition to Ag-specific MHC-peptide complexes and costimulatory molecules (B7.1 and B7.2, signal 2), other signals derived from Ag-primed DCs have been thought to be required for the differentiation of naive Th cells into Th1 and Th2 cells^[72,73,82]. It has been shown recently by us and by others that intestinal helminth infection induces DC activation and up-regulates the DC IL-10 response^[39,83], which may provoke a Th2 and/or T reg dominant response.

HELMINTH INFECTION INDUCES THE DEVELOPMENT OF ALTERNATIVELY ACTIVATED MACROPHAGES

The helminth infection induced-Th2 cytokine response has been suggested to affect macrophages^[84]. Although macrophages share some functional similarities with DCs, macrophages have distinct functions. As discussed above, DCs play an important role in initiating and regulating host immune responses, whereas macrophages, contribute significantly to the effector phase, i.e. elimination of bacteria, and are also thought to be critical mediators of many chronic inflammatory diseases. Distinct phenotypes of macrophages have also been reported. Activation of macrophages by bacterial products (through TLR engagement) or pro-inflammatory stimuli such as Th1 cytokines leads to the development of the classically-activated macrophages. We and others have shown that helminth-induced Th2 cytokines induce a different phenotype, namely, the alternatively activated macrophages^[85-87]. One of the distinctive characteristics of this type of macrophage is the ability to suppress the proliferation of other cells with which they are co-cultured^[88,89]. A recent report suggests that alternative activation of macrophages by IL-4 results in impaired nitric oxide (NO) production and increased expression of the transferrin receptor, supporting intracellular growth of bacteria (*Mycobacterium tuberculosis*)^[90]. Recently, we observed that the exacerbated *C. rodentium*-mediated colitis that develops in helminth-coinfected mice correlates with the marked accumulation of alternatively activated macrophages in colonic LP *via* a STAT6-dependent mechanism^[85]. Functional analysis indicates that these helminth-stimulated macrophages have an impaired ability to effectively control the multiplication of phagocytosed *C. rodentium*. Presumably as a result of the increased bacterial load, these cells also produce increased amounts of TNF- α , a cytokine that has a well-established role in intestinal and other types of inflammation^[91,92]. These observations, therefore, provide evidence to suggest an underlying mechanism for the enhanced bacterial infection and exacerbated bacteria-induced intestinal injury in hosts that are coinfecting with helminth parasites.

CONCLUSION

In many developing countries, exposure to helminth infections and simultaneous infection with other pathogens, such as enteric bacteria, are quite common. Recent evidence indicates that in the developed world, a complete absence of helminth infection may be a predisposing factor for the development of certain immune-mediated disorders^[45]. The emerging evidence indicates the complexity of immune regulation by helminths in host protective immunity and inflammatory diseases. The differences in the effects of helminths on the development and progression of intestinal inflammation observed in various models emphasize

the need for a better understanding of the mechanisms by which helminths modulate host mucosal immunity. It is clear that a more thorough understanding of the complex relationship between the human host and parasitic worms will be required to develop safer, novel and more effective treatments for microbial diseases and immune-mediated disorders such as IBD.

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Roles of galectins in inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD), which is characterized by two forms of intestinal inflammation, Crohn's disease (CD) and ulcerative colitis (UC), is a group of chronic, relapsing, and remitting inflammatory conditions that affect individuals throughout life^[1]. Several factors such as immune imbalance, dysregulated host/microbial interaction, and genetic susceptibility are involved in the pathogenesis of IBD^[2-4]. Experimental IBD models have provided a useful means to dissect the pathogenesis of this disease^[2-4]. Among these models, chronic intestinal inflammation that spontaneously develops in T cell receptor α knockout (TCR α KO) mice shares several features with human UC, e.g. marked increase in autoantibodies such as antineutrophil cytoplasmic antigens and antitropomyosin, predominant Th2 responses and negative association of colitis development with prior appendectomy (resection of cecal patch)^[5-7]. Importantly, B cells and autoantibodies in TCR α KO mice are involved in the regulation of this inflammation^[8-12]. Therefore, a screening approach utilizing autoantibodies present in TCR α KO mice was proposed to have an ability to provide a useful tool in the identification of molecules, which may have a role in the pathogenesis of UC^[13]. Indeed, the screening approach [serological analysis of recombinant cDNA expression libraries (SEREX) for the identification of candidate molecules that are recognized by autoantibodies from TCR α KO mice] has provided us an unexpected opportunity to identify galectin-4 as a potential stimulator of CD4⁺ T cells under intestinal inflammatory conditions^[14,15]. Interestingly, galectin-4 was an unexpectedly discovered carbohydrate-binding protein through our screening approach, emphasizing

Abstract

Protein/carbohydrate interactions through specific protein families termed lectin control essential biological processes. Galectins, a family of animal lectins defined by shared amino acid sequence with affinity for β -galactosides, appear to be functionally polyvalent in a wide range of biological activity. Recent studies have identified immunoregulatory roles of galectins in intestinal inflammatory disorders. Galectin-1 and galectin-2 contribute to the suppression of intestinal inflammation by the induction of apoptosis of activated T cells, whereas galectin-4 is involved in the exacerbation of this inflammation by specifically stimulating intestinal CD4⁺ T cells to produce IL-6. We review how different members of the galectins provide inhibitory or stimulatory signals to control intestinal immune response under intestinal inflammation.

the importance of carbohydrate/protein interactions in the pathogenesis of intestinal inflammation. Indeed, a recent study has demonstrated that an alteration of carbohydrate composition (carboxylated glycans) on macrophages and dendritic cells contributes to the early onset of intestinal inflammation^[16]. Alternatively, carbohydrate/protein interactions also play a regulatory role in the intestinal inflammation as indicated by a suppressive effect of galectin-1 and galectin-2 on this inflammation^[17,18]. We, herein, review recently identified novel roles of galectins in immune responses under intestinal inflammation.

GALECTINS

Several families of glycan-binding proteins or lectins, which include C-type lectins (such as selectin, DC-SIGN, dectin, and serum mannose binding protein)^[19-23], S-type lectins (galectins)^[24-28] and siglecs^[29], have been implicated in a wide variety of immunological functions including first-line defense against pathogens, cell trafficking, cell differentiation and immune regulation. Galectins are a family of 15 members (galectin-1 to galectin-15) characterized by two properties: the ability to bind to lactosamine unit within glycans and the preserved carbohydrate recognition domains (CRD) composed of 130 amino acid residues. The 15 members of galectins are structurally classified into three groups; prototype, chimera-type, and tandem repeat type^[30-32]. Prototype (galectins-1, -2, -5, -7, -10, -11, -13, -14, and -15) is non-covalent homodimers that are composed of two identical CRDs. Only galectin-3 is chimera type that is composed of a CRD linked to a proline-, glycine-, and tyrosine-rich N-terminal domain. Tandem repeat type (galectins-4, -6, -8, -9, and -12) possesses two distinct CRDs. The ability of CRDs to cross-link the lactosamine unit within surface glycoreceptors allows galectins to actively participate in several immune responses. A large body of evidence indicates important roles of galectins in the development and progression of cancer^[30-33]. Recently, compelling evidence has been accumulated regarding the immunoregulatory effects of galectins in inflammatory disorders^[34-36]. We focus on four members of galectins (galectins-1, -2, -3 and -4), which have been studied regarding intestinal inflammation.

REGULATORY ROLE OF GALECTIN-1 IN INTESTINAL INFLAMMATION

Galectin-1 (prototype), which is expressed by a wide variety of cell types, is specifically upregulated on CD4+CD25+ regulatory T cells^[37]. Soluble galectin-1 has been demonstrated to interact with a lactosamine unit of mature core 2 O-glycan that is assembled within some glycoreceptors including CD7, CD43, and CD45, and its cross-linking ability is crucial for the induction of T cell apoptosis^[37-40]. The anti-inflammatory properties of galectin-1 have been evaluated in several models of chronic inflammation and autoimmunity including

autoimmune encephalomyelitis^[41], arthritis^[42], uveitis^[43], hepatitis^[44], and diabetes^[45]. As for the colitis model, galectin-1 expression in the colon is upregulated under an intestinal inflammatory condition that is chemically induced in mouse by rectal administration of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)^[17]. Administration of human recombinant galectin-1 contributes to the reduction of Th1 cytokine (TNF- α , IL-1 β , IL-12 and IFN- γ) release and the suppression of this intestinal inflammation by specifically inducing the apoptosis of effector T cells responsible for production of IFN- γ ^[17]. In addition, a recent study has demonstrated that administration of recombinant galectin-1 also contributes to increase IL-10 production in CD4+ and CD8+ T cells^[46]. These data clearly address the therapeutic potential of galectin-1 to skew the balance from a Th1- toward a Th2-polarized immune response that induces a remission state in the evolution of the ongoing inflammatory disorders.

REGULATORY ROLE OF GALECTIN-2 IN INTESTINAL INFLAMMATION

Galectin-2 (prototype) is expressed by various cells including intestinal epithelial cells. Galectin-2, structurally related to galectin-1, has been demonstrated to be an inducer of apoptosis of activated T cells, although it lacks reactivity to CD7 characteristic for galectin-1^[47]. A recent study has shown that galectin-2 is constitutively expressed mainly in the epithelial compartment of the mouse intestine and binds to lamina propria mononuclear cells^[18]. In acute and chronic dextran sodium sulfate (DSS)-induced colitis, and in a Th1-driven model of antigen-specific transfer colitis, galectin-2 expression was reduced, but could be restored to normal levels by immunosuppressive treatment. Administration of human recombinant galectin-2 induced apoptosis of mucosal T cells and, thus, ameliorated. Furthermore, pro-inflammatory cytokine (IL-6, IL-12p70) release was inhibited by administration of galectin-2. Their study provides evidence that galectin-2, as well as galectin-1, induces apoptosis *in vivo* and ameliorates acute and chronic murine colitis.

ROLE OF GALECTIN-3 IN INTESTINAL INFLAMMATION

Galectin-3 (chimera type) is a multifunctional protein detected in the nucleus, cytoplasm and extracellular matrix of a wide variety of cells. Galectin-3 has the dual role of protecting T cells from apoptosis when present intracellularly while promoting apoptosis when acting on T cells from the extracellular space^[30-32,36]. Regarding intestinal inflammation, a study showed that the titers of anti-galectin-3 autoantibodies were higher in CD patients with low activity index than with active disease^[48]. The pathophysiological significance of the anti-galectin-3 autoantibody in Crohn's disease still remains to be elucidated. The same research group

subsequently showed that expression of galectin-3 was reduced in the intestinal epithelium of CD patients and that colonic epithelial adenocarcinoma cell line HCT-8 cells reduced galectin-3 expression by incubation with TNF- α but not with other cytokines^[49]. It was speculated that galectin-3 was consequently downregulated by enhanced TNF- α production in CD. Another research group confirmed the similar findings^[50]. More recently, soluble galectin-3, which is secreted by colonic epithelial cells, was identified as an activator of lamina propria fibroblasts^[51,52]. The study also indicated that galectin-3 induced NF- κ B activation and IL-8 secretion *in vitro*. Its role in pathogenesis of intestinal inflammation, especially involvement in fibrosis formation of CD, has to be clarified in further studies. In a protein expression profile study of *Enterococcus faecalis*-monoassociated IL-10 KO mice under chronic intestinal inflammation and intestinal epithelial cell lines, galectin-3 expression was reduced in association with the activation of caspase 3, a major executive caspase of apoptosis^[53]. Further studies are needed to address whether galectin-3 plays a pro-inflammatory role or an anti-inflammatory role in intestinal inflammation.

PATHOGENIC ROLE OF GALECTIN-4 IN INTESTINAL INFLAMMATION

Galectin-4 (tandem repeat type) is expressed only in the digestive tract^[54-56] where epithelial cells are responsible for this production^[14,55,56]. Galectin-4 can be secreted from both basolateral and apical sides of the intestinal epithelial cells through a nonclassical secretory pathway. In contrast to galectin-1^[17], intestinal inflammatory conditions do not enhance the galectin-4 expression: there is no significant difference in the expression level of galectin-4 in the epithelial cells from control *versus* inflamed colons^[14]. Interestingly, through a combined screening approach utilizing humoral (SEREX) and cellular immune responses, we have unexpectedly identified galectin-4 as a potential stimulator of CD4+ T cells to exacerbate intestinal inflammation^[14]. Neutralization of galectin-4 activity *in vivo* by administration of the specific antibody suppresses the progression of chronic colitis that spontaneously develops in B cell-deficient TCR α double KO mice^[14], whereas pretreatment with this antibody fails to abolish the development of colitis in these mice (A.M., unpublished observation). These data suggest that galectin-4 contributes to the exacerbation, rather than initiation, of chronic intestinal inflammation. Because it could be predicted that both acute (induction of inflammation) and healing (recovery from inflammation) processes are simultaneously involved in the chronic intestinal inflammation, galectin-4-mediated exacerbation of this inflammation may result from a suppression of the healing process. Indeed, treatment with recombinant galectin-4 delays the recovery from an acute intestinal inflammation that is induced by transient administration of DSS, whereas treatment with anti-galectin-4 antibody

enhances the recovery from this acute inflammation. In contrast, galectin-1, as mentioned above, contributes to the suppression of acute intestinal inflammation^[17]. Galectin-1 (prototype) is structurally characterized by homodimers with identical CRDs, and binds to a lactosamine unit within a mature core 2 O-glycan, whereas galectin-4 (tandem repeat type) consists of two distinct CRDs and possesses a unique carbohydrate-binding specificity as indicated by the capability of interacting with an immature core 1 O-glycan with 3'-O-sulfation^[57]. Therefore, it is highly likely that the binding site (lactosamine unit *versus* core 1) and the structure (prototype *versus* tandem repeat type) are an important determinants of galectin-mediated immune function^[30-32,36]. Galectin-4 specifically stimulates CD4+ T cells, but not other immune cells such as B cells or macrophages to produce IL-6^[14], a well-known cytokine involved in the pathogenesis of not only intestinal inflammation, but also colon cancer^[58-60]. Importantly, only CD4+ T cells that are present in the inflamed, but not non-inflamed, intestine can respond to galectin-4^[14]. Splenic CD4+ T cells even from the diseased mice are unable to respond to galectin-4. These findings are consistent with the binding intensity of galectin-4 to the surface of CD4+ T cells; galectin-4 binding is significantly enhanced on the CD4+ T cells from the inflamed colon as compared to noninflamed colon and spleen. In addition, galectin-4 specifically binds to the lipid rafts on the CD4+ T cells to activate the protein kinase C θ -associated signaling cascade^[14], a common and fundamental pathway in the different types of intestinal inflammation^[61]. Notably, galectin-4 has been demonstrated to interact with lipid rafts of enterocytes as well, and subsequently stabilize the raft formation to generate "superrafts"^[62]. A recent study has found that galectin-4 interacts with carcinoembryonic antigen of colon adenocarcinoma^[63]. Alternatively, it remains obscure which glycosylated receptor(s) on intestinal CD4+ T cells is crosslinked by galectin-4. Production of galectin-4 by colonic epithelial cells is not enhanced under inflammatory condition, whereas the reactivity of CD4+ T cells to galectin-4 is specifically elicited under these conditions. Therefore, it is possible that a specific receptor that is selectively crosslinked by galectin-4 may be expressed on intestinal CD4+ T cells only under inflammatory conditions. However, galectin-4 can bind to the lipid rafts on both CD4+ T cells from inflamed and normal intestines although the binding intensity is much higher on diseased CD4+ T cells^[14]. In addition, expression pattern of the enzymes that are involved in the glycan synthesis is altered by several inflammatory stimuli^[27,34,35]. Therefore, it is more likely that an altered enzyme expression pattern by intestinal inflammatory stimuli results in the further exposure of core 1 O-glycan (a binding partner of galectin-4) on intestinal CD4+ T cells and consequently allows intensified binding of galectin-4 to them. Indeed, our recent studies have found that some glycosylation-associated enzymes, which are involved in the synthesis of core 2 from core 1, are significantly downregulated in the intestinal CD4+

T cells under inflammatory conditions as compared to a state of health (our unpublished observation). These findings provide an insight into an unexpected role of lectin/carbohydrate interaction in the pathogenesis of T cell-mediated chronic colitis.

CONCLUSION

Glycobiology has an exiting impact to molecular biology and clinical fields, given the multifunctional activities of galectins. In this review, we provide novel insights into the role of carbohydrates crosslinked by galectins in the immune responses involved in the pathogenesis of IBD. Different members of the galectin families provide inhibitory or stimulatory signals to control intestinal immune response under intestinal inflammatory conditions. A more thorough understanding of the molecular mechanisms involved in the immunoregulatory functions of galectins is needed before galectin-based therapeutic strategies for IBD can be realized.

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TOPIC HIGHLIGHTS

Emiko Mizoguchi, MD, PhD, Series Editor

Role of mucosal dendritic cells in inflammatory bowel disease

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Abstract

The gastrointestinal innate and adaptive immune system continuously faces the challenge of potent stimuli from the commensal microflora and food constituents. These local immune responses require a tight control, the outcome of which is in most cases the induction of tolerance. Local T cell immunity is an important compartment of the specific intestinal immune system. T cell reactivity is programmed during the initial stage of its activation by professional presenting cells. Mucosal dendritic cells (DCs) are assumed to play key roles in regulating immune responses in the antigen-rich gastrointestinal environment. Mucosal DCs are a heterogeneous population that can either initiate (innate and adaptive) immune responses, or control intestinal inflammation and maintain tolerance. Defects in this regulation are supposed to lead to the two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC). This review will discuss the emerging role of mucosal DCs in regulating intestinal inflammation and immune responses.

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Key words: Dendritic cells; Commensal; Inflammatory bowel disease; Mucosal immunity; Host defence

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INTRODUCTION

The intestinal innate and adaptive immune system has evolved in response to potent stimuli derived from constituents of the commensal microflora. In most cases these local immune responses achieve tolerance to the intestinal microflora and food antigens. Defects of the tightly regulated mucosal immune responses are assumed to result in inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC)^[1,2]. Local T cell immunity is an important compartment of the specific intestinal immune system. T cell reactivity is programmed during the initial stage of its activation by dendritic cells (DCs) that can either initiate (innate and adaptive) immune responses, or control intestinal inflammation and maintain tolerance^[3-5]. DCs reside in mucosal tissues or recirculate in the blood and lymphoid tissues^[6]. The lamina propria of the small and large intestine are effector sites of mucosal tissues. Inductive sites are Peyer's patches (PP), intestinal lymph follicles (iLFs), DC aggregates and mesenteric lymph nodes (MLNs) (Table 1). The local microenvironment influences the phenotype of DCs, a heterogeneous population that can be divided into conventional DCs (CD8 α ⁺CD11b⁻, CD4⁺CD11b⁺, CD4CD11b⁺)^[7,8] and plasmacytoid DCs (B220⁺CD11c^{low}) (Table 2) and are characterized by a remarkable plasticity between DCs^[9]. In the lamina propria of the small and large intestine, DCs are ideally situated to survey the constituents of the commensal microflora and monitor food antigens^[10]. After antigen recognition at peripheral sites DCs migrate to regional draining lymph nodes to initiate (innate and adaptive) immune responses^[11]. This review will discuss the emerging role of mucosal DCs in regulating intestinal inflammation and immune responses.

DCs IN THE SMALL INTESTINE

The lamina propria of the small intestine is populated by CD4⁺CD8 α ⁻ cDCs. Only 2%-5% plasmacytoid DCs reside in the small intestinal lamina propria^[12]. Mucosal DCs can be discriminated into DCs that express CX3CR1 (the receptor for fractalkine/

Table 1 Anatomic compartments of GALT

Inductive/effector sites	Compartment Structure	Abbreviation	Gut segment		
			Small intestine		Large intestine ³
			Upper ¹	Lower ²	
Effector	Lamina propria	LP	+++	+++	+++
	Intraepithelial lymphocytes	IEL	+++	+++	+++
Inductive	Peyer's patch	PP	+	+++	-
	Intestinal lymph follicle	iLF	+	+++	-
	Intestinal lymph aggregate	iLA	+	++	++
	Mesenteric lymph node	MLN	-	-	-

¹Distribution of the described structures in the upper small intestine (duodenum and jejunum); ²Distribution of the described structures in the upper small intestine (ileum); ³Distribution of the described structures in the upper small intestine (ileum). "+++": Very frequent; "++": Frequent; "+": Randomly; "-": Not present.

Table 2 Mucosal DCs and their proposed function

DC ¹		Intestinal compartment ²					Comment ³	Ref.
Lineage	Phenotype	MLN	siLP	cLP	PP	iLF		
cDC	CD4 ⁺ CD8 ⁻ CD11b ⁺ CD11c ⁺	+++	+++	+++	+++	+++	Express CD103 or CX3CR1. Mediate intestinal antigen acquisition. Involved in the RA dependent T _{reg} conversation. Permitting homing of conventional T cells to intestinal tissues by inducing CCR9 and $\alpha\beta\gamma$.	12-14, 47, 86, 99
	CD4 ⁺ CD8 ⁻ CD11b ⁺ CD11c ⁺	+++	-	-	++	+	Prime CD4 T cell responses.	8, 28
	CD4 ⁺ CD8 ⁺ CD11b ⁺ CD11c ⁺	++	-	-	++	++	Prime CD8 T cell responses.	8, 28
	pDC	B220 ⁺ PDCA1 ⁺ CD11c ⁺	+	+/-	+/-	+	Produce type I interferons	10

cDC: Conventional DC; pDC: Plasmacytoid DC; MLN: Mesenteric lymph node; PP: Peyer's patch; cLP: colonic lamina propria; siLP: small intestinal lamina propria; iLF: intestinal lymph follicle. ¹DC lineage; ²Phenotype of the described DC lineage; ³Distribution of the described DC lineage in distinct intestinal compartments.

CX3CL1)^[12] and into DCs that express the integrin α E chain CD103^[13,14]. CD103⁺ DCs originate from Ly-6C^{high}CCR2^{high} monocytes. Conversely Ly-6C^{low}CCR2^{low} monocytes repopulate CX3CR1⁺CD11b⁺ mucosal DCs^[15]. CX3CR1⁺CD11b⁺ DCs directly access the intestinal lumen by extending transepithelial dendrites in a CX3CR1-dependent manner to survey the intestinal lumen^[12], whereas CD103⁺ DCs induce the expression of CCR9 and $\alpha\beta\gamma$ integrin on cognate CD4 and CD8 T cells to facilitate homing of T cells to small intestinal tissues^[13,16] and induce the differentiation of regulatory T cells (T_{regs}) in the absence of exogenous cytokines.

DCs IN THE LARGE INTESTINE

The cLP is populated by CD4⁺CD8⁻ DCs which endocytose and process antigens and induce T cell proliferation. Compared to their splenic counterparts TLR-4, -5 and -9 expression by colonic CD4⁺CD8⁻ DCs is low^[17]. Only few CD8⁺ and plasmacytoid DCs are found in the cLP^[17]. In human biopsies from colonic tissues, CD3⁺CD14⁺CD16⁺CD19⁺CD34⁺ DCs with an immature state with low TLR-2 and -4 expressions were observed^[18]. In addition the presence of CD83⁺ and DC-SIGN⁺ DCs was described^[19]. Human and mouse mucosal DCs are assumed to be less responsive to microbial-derived TLR-ligands compared to

spleen or blood born DCs^[17]. Despite the functional subspecifications of DCs, DCs are characterized by a remarkable plasticity between DCs which is influenced by the local environment, the antigen itself or the activation state of the DC^[9,20].

DCs IN PPs

PP, iLF, CP and DC clusters of the small and large intestine belong to the gut associated lymphoid tissues (GALTs)^[6], secondary lymphoid structure, which lack in contrast to lymph nodes the afferent lymph and are located in close proximity to the intestinal epithelium. In PP the subepithelial dome regions beneath the follicle-associated epithelium can be discriminated from follicular and interfollicular regions, which serve as inductive sites where immune responses are primed. Specialized epithelial cells, the M cells, deliver luminal antigens to DCs located in the subepithelial dome regions beneath the follicle associated epithelium^[21]. The subepithelial dome regions are populated with CD8 α ⁺CD11b⁺B220⁻ DCs and with CD8 α ⁺CD11b⁺B220⁻ DCs^[22,23]. By expressing high concentrations of the chemokines CCL20, CXCL16 and CCL9, the follicle-associated epithelium creates a specific micromilieu that allows DCs to selectively migrate towards the follicle associated epithelium^[24,25]. Upon pathogen challenge

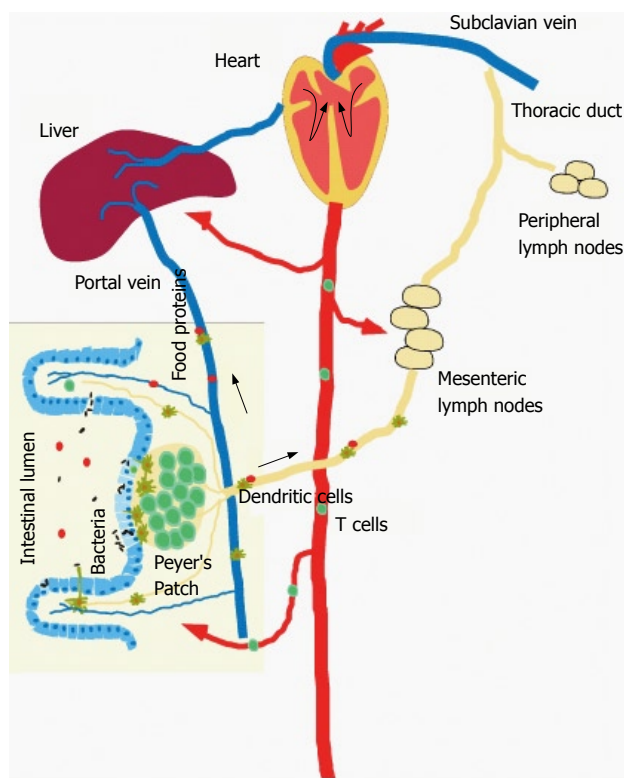


Figure 1 Bacterial and food antigens are continuously surveyed by the mucosal immune system, processed and transported via the lymph to mesenteric lymph nodes or via the portal vein to the liver. DCs present processed luminal antigens to naïve T cells to achieve tolerance or to initiate host defence to pathogens.

CCR6⁺ DCs which are located in the interfollicular regions are recruited towards the follicle-associated epithelium to process microbial antigens, and to facilitate rapid local adaptive immune responses^[23]. In the absence of CCR6 in CCR6^{GFP/GFP} mice CX3CR1⁺ DCs that lack CCR6 expression reside in the follicle-associated epithelium^[23,26]. In the interfollicular regions CD11b⁺ CD8 α ⁺ and CD11b⁺ CD8 α ⁺ DCs as well as plasmacytoid DCs are found that produce IL-12 and IL-10, and induce the differentiation of IFN- γ secreting TH1 cells, whereas the CD11b⁺ subsets produce low IL-12 and high IL-10 levels and prime IL-10 producing T cells^[22,27]. CD8⁺33D1⁺ DCs, but not CD8 α ⁺DEC205⁺ DCs are specialized to present processed antigens in a MHC class II dependent manner to CD4 T cells^[28]. Reoviral antigens are sampled and processed by CD11b⁺CD8 α ⁺ DCs to initiate TH1 adaptive immune responses likely resulting in protective host defence to viral antigens^[29].

DCs IN INTESTINAL LYMPHOID TISSUES

Intestinal lymphoid aggregates (iLAs) can be discriminated from PP, which comprises iLF and CP that are closely associated with the epithelial lining in the small and large intestine^[30]. CP were defined as tiny aggregates of c-Kit⁺IL-7R⁺ cells^[31], whereas iLFs are constituted by solitary B follicles that are localized in the anti-mesenteric regions of the intestinal wall, and contain small numbers of mature T lymphocytes, but

also c-Kit⁺ and IL-7R⁺ cells^[32-34]. By analyzing large numbers of such aggregates, the classification of iLA into CP and iLF was recently challenged showing that most of them display properties intermediate between CP and iLF and were termed small intestinal lymphoid tissues (siLT)^[35]. In the large intestine, subepithelial DC aggregates locate in the regions of cLP where inflammation develops^[36]. DC aggregates are formed by CD8 α ⁺, CD8 α ⁺ and B220^{lo} DCs. DCs with high MHC II expression, but low expression of the costimulatory molecules CD40, CD80 and CD86 extend transepithelial dendrites into the intestinal lumen^[37]. During colitis induced by adoptive transfer of CD45RB^{hi} CD4 T cells into immunocompromised RAG mice, FoxP3 regulatory T cells accumulate in colonic DC aggregates producing TGF- β suggesting that DC aggregates are important structures to regulate inflammation^[37].

DCs IN MLN

Luminal antigens are sampled by lamina propria DCs and by DCs located in PP, CP, iLF and DC clusters at the base of intestinal villi. The DCs transport the sampled antigens *via* the lymph to MLN, where naïve T cells are primed and adaptive immune responses are induced^[38] (Figure 1). In MLN, blood-born DCs and DCs that originate from PP or the lamina propria are present^[6]. In MLN, blood-born DCs are assumed to express CD8 α ^{hi}CD11b⁺ α L^{int} β 7^{int} and CD8 α ⁺CD11b^{hi} α L^{lo} β 7^{lo} DCs, whereas DCs derived from mucosal sites are characterized by CD8 α ^{int}CD11b⁺ α L^{lo} β 7^{hi} and CD8 α ⁺CD11b^{hi} α L^{lo} β 7^{hi} expression^[39], allowing the discrimination of DCs of distinct origins. CCR7 expression guides lamina propria DCs to MLN^[40]. Blood-born DCs enter the MLN *via* interactions with mucosal addressin cellular adhesion molecule (MAdCAM-1) and peripheral node addressin (PNAd) expressed by high endothelial venules (HEVs)^[41]. Apoptotic cell bodies derived from intestinal epithelial cells (iECs) are phagocytosed and transported to MLN to induce tolerance against self-antigens^[42]. The depletion of MLN results in defective oral tolerance. Lamina propria DCs are continuously carrying commensal bacteria to the MLN^[43], which can be further potentiated by stimulating DCs with TLR-7/8 agonists^[44]. IgA production is induced that is supposed to limit the dissemination of commensal bacteria further than the MLN preventing effectively systemic infections^[45].

DCs IN INTESTINAL ANTIGEN ACQUISITION PATHWAYS

Intestinal DCs play a key role in monitoring the intestinal lumen by continuously sampling and processing luminal antigens^[46,47]. There are different pathways by which luminal antigens can gain access in the lamina propria^[48]. In all pathways, mucosal DCs are supposed to play key roles. Restricted to the ileum DCs extend transepithelial dendrites in a CX3CR1 mediated manner to survey the

intestinal lumen to continuously survey the commensal microflora and to monitor food antigens^[12,49]. M cells located in the follicle associated epithelium of PP are specialized epithelial cells which gained the ability to translocate luminal antigens *via* the intestinal barrier and deliver luminal antigens to DCs located in the subepithelial dome regions of PP^[21]. Upon pathogen challenge, CCR6⁺ DCs are recruited to the dome regions to process pathogens, and to trigger rapid local adaptive immune responses^[23]. As well CD11b⁺CD8 α ⁻ CD11c⁺ interfollicular DCs process antigens derived from luminal reovirus^[29]. M cells within the small intestinal villous epithelium are able to translocate pathogens *via* the epithelial barrier to DCs located in the lamina propria^[50]. The intestinal epithelium by itself plays an important role in sensing the luminal microenvironment. The intestinal epithelium express Toll-like receptors, such as TLR2^[51], TLR-4^[52], TLR-5^[53] and TLR-9^[54] to recognize microbial derived pathogen associated molecular patterns (PAMPs) for the regulation of protective innate immune responses. As well upon LPS-stimulation iECs express the intracellular pathogen recognition pattern NOD1^[55] and NOD2^[56,57], in which mutations are associated with an increased susceptibility for CD^[58,59]. IECs directly participate in intestinal antigen uptake pathways by delivering antigens or exosomes to lamina propria DCs. Secreted IgG binds to cognate luminal antigens to form IgG/antigen complexes that are recycled by neonatal FcRn receptors and delivered to lamina propria DCs^[60,61]. IECs also express MHC II and may present luminal antigens to CD4 T cells. After luminal exposure with ovalbumin (OVA), OVA is taken up at apical and basolateral surfaces of the epithelial cells. Then, it enters the early endosomes and may be delivered to late endosomes where it is processed in the presence of MHC II, and presented in the context of class II to T cells^[62]. It needs to be dissected in future work whether all or specific luminal antigen acquisition routes are linked to the induction and constant renewal of tolerance and to the development of protective immune responses. DCs are assumed to play key roles in all luminal antigen acquisition routes, and are supposed to represent a major factor for the processing and presentation of orally delivered antigens and the induction of tolerance.

DC MEDIATE INNATE AND ADAPTIVE IMMUNE RESPONSES TO THE COMMENSAL FLORA

The gastrointestinal immune system faces the tremendous challenge to deal with potent stimuli for the innate and adaptive immune system derived from the commensal microflora and food antigens. DCs are sentinels of the mucosal immune system to survey the constituents of the luminal commensals and to trigger host responses to pathogens^[63]. It is thought that gastrointestinal diseases, such as IBDs are results of deregulated immune responses to the commensal microflora^[1].

Although germ-free (GF) mice and rats generate alloreactive T cell responses^[64] and cellular immunity to certain bacteria (after monocolonization)^[65,66], the cellularity of their immune system is greatly reduced^[67-69]. In this regard, the numbers of IL-17A producing TH17 cells is reduced in the lamina propria of GF animals^[70]. Because commensal flora is a major driving force of the homeostatic proliferation of naïve T cells in the periphery, the reduced cellularity in the immune system of GF mice may be the result of the deficient peripheral expansion of recent thymic T cell emigrants^[71,72]. When GF mice are fed bacterial carbohydrate polysaccharide A (PSA) CD4 T cells are activated resulting in a TH1 response imprinted by DCs^[72]. After conventionalization of GF rats OX62⁺ DCs increased, whereas CD4⁺ DCs located in the follicle associated epithelium decreased indicating that CD4⁺ DCs phagocytose microbial derived antigens, relocate to the follicular regions, and elicit rapid immune responses to the microflora to achieve tolerance^[68]. Furthermore, it has been described that the inducible isoform of nitric oxide synthase (iNOS) expressing DCs is markedly reduced in GF or MyD88-deficient animals indicating that the commensal flora is required for the accumulation of iNOS expressing DCs at mucosal sites^[73]. In iNOS^{-/-} animals serum IgA levels are reduced suggesting that iNOS expressing DCs play a role in IgA class switching required to prevent the uncontrolled dissemination of the commensal flora into the host.

HEMATOGENOUS DISSEMINATION OF INTESTINAL ANTIGENS

Although the priming of T cells mainly occurs in draining lymph nodes, antigen-rich blood from the small and large intestine is transported to the liver *via* the portal vein, and pressed through a network of sinusoids and scanned by antigen-presenting cells and lymphocytes in the liver^[74,75] (Figure 1). One third of liver cells are constituted by non-parenchymal cells (NPCs), which include liver sinusoidal endothelial cells (LSECs), Kupffer cells, biliary cells, stellate cells (Ito or fat-storing) cells and lymphocytes^[74,75]. Resident APCs in the liver are Kupffer cells, LSEC^[76], Ito cells^[77] and DCs^[75]. Plasmacytoid B220⁺CD11c⁺ DCs as well as B220⁺CD11c⁺ DCs are found. The latter can be further divided into major immature (CD40^{lo} CD80^{lo} CD86^{lo} MHC class II^{lo}) CD11c^{int} and minor mature (CD40^{hi} CD80^{hi} CD86^{hi} MHC class II^{hi}) CD11c^{hi} DCs^[78]. Further, the presence of CD8 α ⁺CD11b⁻ and CD8 α ⁻CD11b⁺ DCs has been reported^[78]. The continuous exposure of resident APCs with the bacterial cell wall derived LPS promotes the induction of CD4 regulatory T cells and may explain the dominance of IL-10 in the liver^[79,80]. If Kupffer cells are depleted by gadolinium chloride treatment, liver tolerance becomes impaired raising the possibility that the liver is increasingly recognized as an innate and adaptive immune organ^[81].

DCs IN ORAL TOLERANCE INDUCTION

Mucosal DCs play a key role in the development of oral tolerance, a phenomenon, in which systemic immune responses to a defined peptide/protein are blunted, when the same protein has been orally fed before the rodents were systemically challenged^[82]. The triggering of oral tolerance in patients with IBD is impaired^[83]; however, the exact mechanism has not been determined. Mucosal DCs are critical for the induction of oral tolerance as shown by studies, in which flt-3 ligand was injected in mice leading to the expansion of DC subsets^[84]. After the expansion of the intestinal DC pool, enhanced oral tolerance was observed. Because oral tolerance can be transferred by adoptive transfer of T cells into recipient mice, interactions between DCs and T cells seems to be essential for the development of oral tolerance, in which CD4 CD25 FoxP3 regulatory and IL-10 and TGF- β secreting TH3 cells that suppress systemic immune responses are primed^[85]. When spleen DCs are compared to mucosal DCs, the mucosal DCs are more efficient in inducing FoxP3 expression than spleen DCs in the presence of TGF- β ^[86,87]. Further analyses demonstrated that CD103⁺ DCs are able to induce the differentiation of T_{reg} cells *via* the production of the Vitamin A metabolite, retinoic acid, in presence of TGF- β , which in addition results in the recruitment of T cells and B cells to intestinal tissues^[88-90]. Blocking of TGF- β abrogates the ability of CD103⁺ DCs to induce T_{reg} cells^[91,92]. However, parallel studies have indicated that exogenous TGF- β has not to be added to this system in order to obtain FoxP3 expressing T_{reg} cells^[86,87] raising the possibility that gut DCs activates latent TGF- β present in mucosal tissues. In this regard, the integrin $\alpha_v\beta_8$ expressed by DCs is required to activate TGF- β *in vivo*. Mice, in which DCs lack $\alpha_v\beta_8$, develop IBD and autoimmunity^[93].

Oral tolerance is not impaired in PP-deficient mice demonstrating that lamina propria DCs play a key role in the development of oral tolerance by sampling the luminal content, and transporting antigens to the MLN^[94]. After depletion of the MLN, the development of oral tolerance is reduced indicating that the MLN are a major site for priming tolerance^[95]. Interestingly, it has been suggested, that intestinal self antigens can be presented by lymph node stroma cells beyond the MLNs^[96]. It also may depend on the presence of certain pathogens within the lumen, because *Heligmosomoides polygyus* infection correlates with DC activation and IL-10 expression^[97]. This provides evidence that the studies of intestinal microbial responses not only require the recognition of region- and compartment-specific immune responses, but the consideration of the interplay of different commensals and pathogens in modulating mucosal immune responses.

DC DEPENDENT T_{reg} CELL CONVERSION

T_{reg} cells play a key role for the development of oral tolerance, and the regulation of intestinal inflammation

triggered by the intestinal microflora. CD103⁺ but not CD103⁻ DCs purified from GALT-induced generation of FoxP3 cells *in vitro*^[98]. T_{reg} cells can be distinguished from other T cell populations by the surface expression of the α chain of the high affinity IL-2 receptor (CD25), and the transcription factor FoxP3 that is essential for T_{reg} development. Deadly autoimmunity is prevented by T_{reg} cells as exemplified in X-linked immunodeficiency, polyendocrinopathy, enteropathy (IPEX) syndrome in humans^[98]. The conversion of T_{reg} cells in the GALT can be prevented by inhibitors of retinal dehydrogenases, an enzyme that is highly expressed by CD103⁺ DCs, which converts retinol (vitamine A) into retinoic acid^[86,87,91,99]. Retinoic acid binds to nuclear retinoic acid receptors that upon ligation can inhibit the activity of activating protein-1 (AP-1), a transcription factor, which can interfere with nuclear factor of activated T cells (NFAT)-FoxP3 complex^[100,101]. However, the exact retinoic acid dependent signal transduction pathways required for the conversion of T_{reg} cells remains to be elucidated. Retinoic acid production by CD103⁺ DCs also induce the up-regulation of the integrin $\alpha_4\beta_7$, and the CC chemokine receptor CCR9 which binds thymus-expressed chemokine (TECK) permitting the accumulation of T_{reg} cells intestinal tissues^[13]. The retinoic acid dependent conversation of T_{reg} cells depends on the presence of TGF- β , a cytokine that, in the presence of IL-6 and IL-23, can also induce the generation of TH17 cells^[102] (Figure 2). A small subset of naïve T cells in the small intestine co-express the transcription factors FoxP3 and ROR γ ^[103]. High expression of TGF- β represses IL-23 receptor expression and favours the generation of FoxP3 T_{reg} cells, whereas low TGF- β expression in concert with IL-6, IL-21 and IL-23 relieves FoxP3 mediated ROR γ T inhibition promoting TH17 cells^[103]. TH17 cells accumulate in the lamina propria of patients with IBD indicating that TGF- β can induce regulatory and pro-inflammatory T cell subsets^[104].

DCs IN IBD

Various animal models have provided insights that mucosal DCs play a key role in IBD. However, the specific function of certain DCs are unknown and needs to be determined in future work, which will provide information on mechanisms leading to IBD and limiting intestinal inflammation to achieve protective mucosal immune responses. In agreement with animal models DCs accumulate at sites of inflammation in patients with IBD. It was found that the pathogen recognition receptors TLR-2 and -4 as well as the activation/maturation marker CD40 are upregulated by intestinal DCs derived from patients with CD^[18]. Furthermore, increased numbers of TNF- α producing MDC8⁺ monocytes, which may be precursors of mucosal DC populations, were found in patients with IBD and, hence, the treatment CD patients with anti-TNF- α antibodies resulted in reduced DC activation^[105,106]. In inflamed tissues DCs are matured and increased in

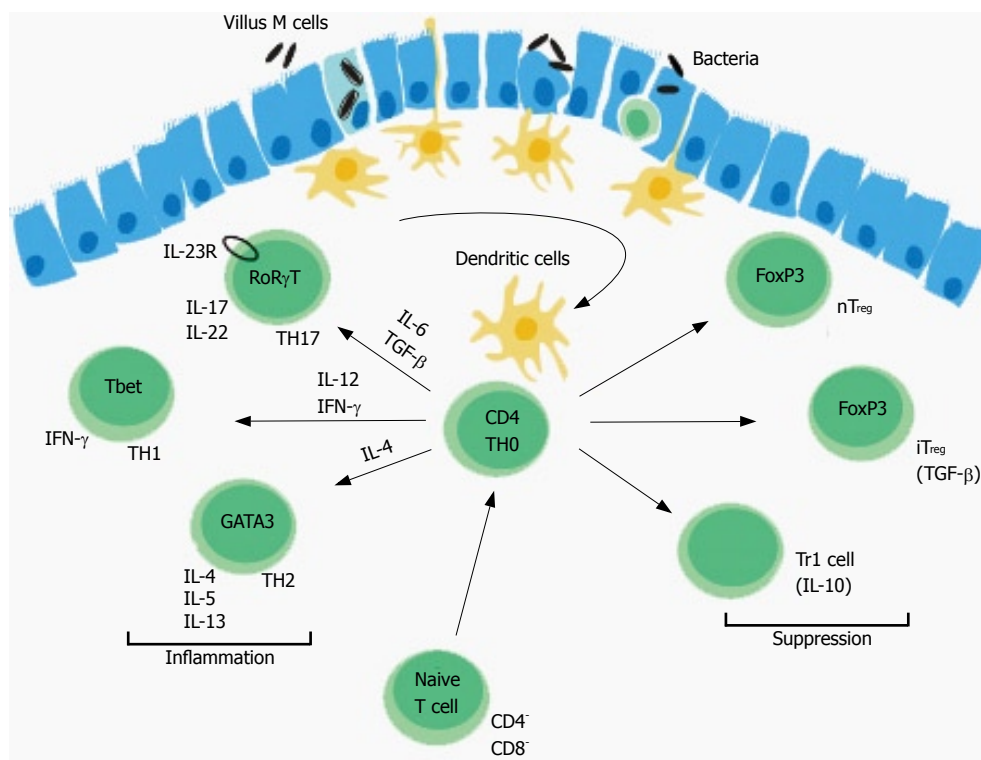


Figure 2 DCs continuously survey the intestinal lumen, phagocytose and process luminal antigens and present them to T cells, which will differentiate in presence of IL-6 and TGF- β to IL-17A and IL-17F producing TH17 cells, in presence of IL-12 and IFN- γ into TH1 cells or in presence of IL-4 into TH2 cells. nTreg, natural occurring regulatory T cell; iTreg, inducible regulatory T cells; Tr1, regulating T cell.

numbers. The CD83⁺CD80⁺DCSIGN⁺ DC subsets produce the cytokine IL-12 and IL-18, which promote TH1 development^[119]. Also, in the peripheral blood and in the lamina propria of patients with CD or UC the numbers of CD86⁺CD40⁺ DCs are increased. In addition, DCs generated *ex vitro* from peripheral blood monocytes of IBD patients show increased abilities to stimulate immune responses^[107-109]. Mice studies indicated that DCs isolated from inflamed colonic tissues and DCs located in the terminal ileum, which continuously sample commensal bacteria produce IL-23, but little IL-12^[110,111]. It was discovered that IL-10 KO mice that spontaneously develop colitis are protected from colitis when bred on IL12p19, but not on IL-12 p35-deficient mice. In similar studies, *Helicobacter hepaticus* infected immunocompromised RAG^{-/-} bred on IL-12 p19 deficient mice, but not on IL-12 p35 deficient background mice were protected from colitis indicating that DC derived IL-23 plays a major role in intestinal pathology^[112,113]. The formation of granulomas, histological characteristics of CD, depends on the release of IL-23 by DC-like cell types that are characterized by CD11c and F4/80 expression^[114]. Genome-wide association studies showed associations between CD and UC patients, and a gene encoding a subunit for the IL-23 receptor suggested a major role of IL-23 in the pathogenesis of IBD^[115,116]. IL-23 seems to be essential for the expansion and maintenance, but not for the initial induction of IL-17 producing CD4⁺ T cells (TH17) cells^[117]. Studies in which TH1 and TH17 cells were generated co-cultures, in which naïve T cells were cultured with fecal extracts pulsed DCs, and in the presence of TH1 or TH17 promoting cytokines indicated that TH17 cells are more pathogenic than TH1

cells^[118]. Recent published observations report that colitis induced by transfer of IFN γ -deficient T cells in RAG^{-/-} mice is associated with elevated numbers of TH17 cells in the lamina propria^[70]. The adoptive transfer of IL-17F, but not IL-17A deficient CD4⁺ T cells ameliorated the IBD in the transfer model^[119,120]. Interesting findings implicated that the IL-1 and IL-23 dependent priming of TH17 effector cells required a NOD2 dependent pathway, and that monocyte derived DCs from CD patients with mutated NOD2 failed to efficiently activate TH17 effector cells^[121]. In this regard the TH17 cytokine IL-22 mediates mucosal defence to bacterial pathogens, and ameliorates chronic colitis in the TCR α KO model by stimulating mucus production and goblet cell restitution under inflammatory conditions^[122]. In addition IL-22, which is released by T and DCs, act together with IL-17 to clear bacterial infections at mucosal sites^[123,124]. When conventional DCs are depleted in a CD11c DTR transgenic animal system by diphtheria toxin applications, the severity of colitis is suppressed in the dextran sodium sulfate (DSS) colitis model^[125]. Furthermore, in mice with an iEC specific deletion of IKK β failed to clear *Trichuris muris* infection characterized by severe intestinal pathology^[126]. In these mice an increased accumulation of DCs at mucosal sites was observed that produce IL-12/23 p40 and TNF- α . In addition, an accumulation of IFN- γ producing TH1 and IL-17 producing TH17 cells in the MLN was found. Specific depletion of NEMO (IKK γ) or of both IKK α and IKK β is essential for the activation of NF- κ B activation induce IBD^[127]. Constitutive NF- κ B activation in IECs by commensal flora may condition DCs to prevent tissue inflammation. Thymic stromal lymphopoietin (TSLP) produced by epithelial cells is involved in the

conditioning of DCs to prime less harmful TH2 and T_{reg} responses^[126,128]. Together, these data suggest that DCs are conditioned by iECs to promote immunosuppressive T cell responses. However, DCs and their precursors are sensitive to proinflammatory activation signals, which could help to participate into the long persistence of local T cell activation patterns promoting IBD.

CONCLUSION

Mucosal DCs may have several functions in the mucosal immune system to accomplish tolerance and to maintain homeostasis. Tolerance to intestinal self antigens, oral antigens and the commensal flora is achieved by interactions of DCs with regulatory and effector T cells. DCs are also involved in triggering deleterious T cell responses to the endogenous microflora being the basis of IBD. Mucosal DCs express the integrin alpha E (CD103) or the receptor for fractalkine/CX3CR1. CX3CR1 expressing DCs are involved in luminal antigen recognition pathways, whereas CD103 DCs metabolize vitamin A to retinoic acid and are involved in the conversion of T cells to regulatory T cells. Genetically engineered mouse models, and cellular approaches will be increasingly available to study the biology of CD103 and CX3CR1 DCs in immune responses to the commensal flora, and their role in initiating and regulating intestinal inflammation. DCs maintain intestinal homeostasis allowing the peaceful coexistence with the endogenous microflora. The discovery of specific DCs associated with luminal antigen acquisition and oral tolerance will allow developing strategies for targeting defined antigen acquisition routes to design therapeutic treatments for patients with IBD.

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Toll-like receptors in inflammatory bowel disease-stepping into uncharted territory

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Abstract

Ulcerative colitis and Crohn's disease are chronic relapsing-remitting inflammatory processes of the intestinal tract. The etiology of these diseases is currently unknown. However, inflammation is hypothesized to result from inappropriate activation of mucosal immunity by luminal antigens in genetically susceptible individuals. Toll-like receptors (TLRs) are a family of transmembrane proteins that act as microbial pattern recognition receptors. They are crucial initiators of innate immune responses. The role of TLRs in the pathogenesis of inflammatory bowel disease (IBD) has not been fully elucidated. In this review, we aim to analyze the available data connecting individual TLRs to intestinal inflammation and IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is comprised of two major forms of chronic inflammation of the gastrointestinal tract, ulcerative colitis (UC) and Crohn's disease (CD). These two entities differ in their location (colon only *vs* the whole length of the intestinal tract), pattern of distribution (continuous *vs* patchy), depth of involvement (mucosal *vs* transmural) and histology (crypt abscesses *vs* granulomas). The onset of IBD typically occurs in the second and third decades of life, and a majority of affected individuals progress to relapsing and chronic disease^[1].

The etiology of IBD is currently unknown. Inflammation is hypothesized to result from inappropriate activation of mucosal immunity by environmental factors in genetically susceptible individuals^[2]. There is strong evidence to support the role of intestinal microflora in the pathogenesis of IBD. Mice raised under germ-free conditions do not develop spontaneous colitis in several experimental models^[3]. Additionally, antibiotic treatment and probiotic bacteria were shown to induce remission in IBD patients^[4-6]. Inappropriate activation of innate immunity is the other arm involved in the pathogenesis of IBD^[7,8]. Activation of innate immunity relies at least partially on recognition of conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)^[9]. There are two major families of PRRs known as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors.

TLRs

There are currently 11 known mammalian TLRs. They are transmembrane receptors that are found either on the cell membrane (TLR1, 2, 4, 5 and 9) or on intracellular organelles (TLR3, 7 and 8)^[10,11]. TLRs are expressed throughout the gastrointestinal (GI) tract on intestinal epithelial cells (IECs), myofibroblasts, enteroendocrine cells, and on immune cells within the lamina propria, such as T cells, and dendritic cells (DCs)^[12-16]. Extracellular domains of TLRs consist of leucine-rich repeats (LRRs), whereas their intracellular component contains a TIR (Toll/IL-1 receptor) domain, exhibiting homology with the interleukin-1 receptor (IL-1R) superfamily. Most TLR signal *via* the MyD88

adaptor (TLR1, 2, 4, 5, 6, 7, 8 and 9), whereas TLR3 signaling activates an alternative “MyD88-independent” pathway. TLR4 is the only receptor known to activate both MyD88-dependent and -independent pathways^[17]. Ligand binding to TLRs initiates signaling cascades that activate NF- κ B, MAPK, and interferon response factors. In this review, we will address the role of the TLR family in the pathogenesis of IBD.

TLR4

The Toll-like receptor 4 (TLR4) gene is located on the long arm of human chromosome 9. Its ligand is lipopolysaccharide (LPS), and signal initiation requires the presence of CD14. In mouse models, TLR4 is involved in regeneration of IECs. TLR4 KO and MyD88 KO mice show impaired mucosal healing, and disturbed barrier function in response to administration of the colitis inducing dextran sulfate sodium (DSS) which leads to an increase in intestinal bleeding, colonic damage, bacterial translocation and to increased mortality. A similar aggravating effect of DSS was observed when natural ligands of TLR4 were eliminated either by broad spectrum antibiotic treatment or by raising the mice in a germ-free environment^[18]. TLR4 signaling was shown to play a role in the initiation of intestinal inflammation. Treatment with CRX-526, a TLR4 antagonist, inhibited the development of moderate-to-severe colitis in MDR 1a-deficient mice, and TLR signal abrogation by MyD88 KO prevented development of spontaneous colitis in IL-10 KO mice^[19]. Human IECs normally express TLR3 and TLR5, whereas TLR2 and TLR4 are only minimally expressed^[20]. However, TLR4 expression is upregulated in both CD and UC. In pediatric IBD patients, higher levels of TLR4 mRNA and protein were found in the inflamed colonic mucosa, but not in non-inflamed controls^[21].

Epidemiological studies show an association between TLR4 polymorphism and susceptibility to IBD. In a German cohort, the CD14 promoter 1-260C>T single-nucleotide polymorphism (SNP) was associated with UC, but not CD, while the opposite was found in a Hungarian cohort. No association with IBD of the TLR4 896A>G SNP was found in either cohort^[14]. In a Belgian study, the allele frequency of the TLR4 A299G polymorphism, affecting the extracellular domain of TLR4 that is associated with an abrogated response, was significantly higher in CD (11% *vs* 5%, $P = 0.004$ in one cohort and 12% *vs* 5%, $P = 0.007$ in another cohort) and in UC patients (10% *vs* 5%, $P = 0.027$) compared with controls^[22]. The same SNP was exclusively related to CD in another study^[23] and a third study found no association of this SNP with IBD^[24]. Another TLR4 polymorphism, T399IL, was exclusively associated with UC, and not with CD^[25].

TLR5

TLR5 is highly expressed in colonic epithelial cells (CECs). The bacterial ligand of TLR5 is flagellin, which is present on most motile bacteria. Expression of TLR5 appears to be basolateral in healthy individuals, and CECs in the

intact colon do not respond to flagellin. It is suggested that the response of CECs to flagellin is specifically elicited under inflammatory conditions with epithelial barrier disruption^[26,27]. Consistent with these findings, rectal administration of flagellin to control mice did not elicit an inflammatory response, but was able to aggravate DSS-induced colitis^[26]. Interestingly, flagellin derived from *Salmonella* species, but not commensal bacteria was able to stimulate proinflammatory chemokines secretion by IECs^[28]. In CD patients, tolerance to commensal-derived flagellin is lost and serum reactivity to flagellin can be demonstrated^[29]. Additionally, CD patients carrying a susceptibility NOD2 mutation exhibit an enhanced flagellin reactivity which was independently associated with distinct CD phenotypes^[30]. TLR5 KO mice develop spontaneous colitis, and a marked elevation in pro-inflammatory cytokine levels. This colitis is mediated *via* TLR4 signaling as TLR5 and TLR4 double KO fail to develop spontaneous colitis^[31].

Data connecting TLR5 polymorphism to IBD is limited. Recently a TLR5-stop polymorphism in which a point mutation at nucleotide 1174 generates a stop codon rendering TLR5 inactive was described^[32]. It is a relatively common polymorphism with a 5% allele frequency. Healthy subjects carrying the TLR5-stop SNP have significantly lower levels of flagellin-specific IgG and IgA, but similar levels of total and LPS-specific immunoglobulins. Moreover, it was observed that the carriage rate of the TLR5-stop SNP was significantly lower in CD patients as compared with unaffected relatives and unrelated controls^[33].

TLR9

Unmethylated cytosine-guanosine dinucleotides (CpG), which are frequently recognized in the DNA of bacteria and viruses, are the ligands for TLR9^[34,35]. The role of TLR9 signaling in pathogenesis of IBD is under intense investigation.

It was reported that intragastric and subcutaneous administration of CpG oligodeoxynucleotides (CpG-ODNs) reduced the severity of DSS and TNBS-induced acute colitis and of chronic colitis in IL-10 KO mice^[36]. It was further shown that the beneficial therapeutic effect of probiotic bacteria in murine colitis models was mediated *via* the effect of CpG on intact TLR9 signaling^[37]. Later, it was suggested that type I interferons may act as immunomodulatory effectors of the TLR9 pathway^[38]. Further support for the immunosuppressive role of CpG-ODNs came from studies in which CD4+CD62L+ T lymphocytes from CpG-ODN pretreated donor mice were unable to induce colitis in recipient SCID mice in a transfer model, and were able to suppress CD4+ T cell-mediated colitis when co-transferred. Furthermore, CD4+CD62L+ cells from TLR9 deficient mice induced a significantly more severe colitis in SCID recipients than cells from controls^[39]. These data would suggest that TLR9 signaling suppresses intestinal inflammation.

In contrast, a pro-inflammatory effect of TLR9 signaling in colitis models has also been demonstrated.

Intraperitoneal administration of CpG-ODN increased the severity of DSS-induced acute and chronic colitis^[40]. Induction of DSS colitis in TLR9-deficient mice resulted in markedly reduced intestinal inflammation and proinflammatory cytokine production. Additionally, treatment with adenoviral ODN, known to block CpG effects, resulted in a significant amelioration of colitis^[41].

Recently, a novel mode of TLR9 pathway regulation was described which could, at least in part, explain some of the above mentioned discrepancies. Basolateral TLR9 signaling was shown to activate the NF- κ B pathway in CECs, whereas apical TLR9 signaling inhibited its activation by inducing accumulation of ubiquitinated I κ B in the cytoplasm. Moreover, apical stimulation of TLR9 prevented activation of NF- κ B signaling by other TLRs^[8]. These data suggest that different routes of CpG-ODN delivery (intraluminal *versus* systemic) may result in anti- or pro-inflammatory effects, respectively. Additionally, this report provides a possible explanation of the divergent effects of CpGs on colitis depending on the time-point of application. Administration of CpG-ODNs when the epithelial barrier is intact results in a protective effect, while the same administration, when the epithelium is disrupted leads to aggravation of inflammation^[42].

The effect of CpG-ODN stimulation in IBD was assessed using *ex vivo* colonic mucosal biopsies from active UC patients and healthy controls. CpG-ODNs significantly inhibited colonic TNF- α and IL-1 β generation in a TLR9-dependent manner in UC, and not in controls^[43].

In epidemiological studies, the frequency of the -T1237C SNP of the TLR9 promoter region was significantly increased in patients with Crohn's disease as compared with controls^[44].

TLR 1, 2, 6

TLR2 is required for recognition of Gram positive and mycobacterial PAMPs including bacterial lipopeptide, lipotechoic acid (LTA) and peptidoglycan (PGN). Following ligand association, TLR2 and TLR6 \pm TLR1 form hetero-dimers that may control signal specificity and enhance signal transduction^[45, 46].

TLR2 KO mice display increased susceptibility to DSS-induced colitis. In mice, TLR2 stimulation effectively augments tight junction barrier assembly against stress-induced damage through the activation of PI3K/Akt pathway. Oral treatment of mice with the TLR2 ligand Pam3CSK prior to DSS colitis induction significantly suppressed mucosal inflammation and apoptosis and restored epithelial integrity^[47]. In contrast to its anti-inflammatory effect, another report suggested that Pam3CSK administration may actually augment colitis. Suppression of colitis by regulatory T lymphocytes in the CD45RB^{high} T cell transfer model was significantly delayed when the cells were pretreated with Pam3CSK^[48].

Baseline expression level of TLR2 on enterocytes was reported to be low, but its levels increased with inflammation. Immunohistochemical analysis showed

either a significant increase or no change in TLR2 expression in the terminal ileum of patients with inactive and active IBD compared to controls^[20, 49]. Another study showed that monocytes isolated from patients with active IBD had higher expression levels of TLR2 on their cell surfaces, and a significantly increased TNF- α production in response to TLR2 agonist stimulation as compared to inactive patients and healthy controls^[50]. It was recently suggested that NOD2, which is mutated in 15% of CD patients, and was the first CD susceptibility gene to be discovered, is involved in TLR2 signal regulation. Specifically, it was shown that muramyl-dipeptide (MDP), which is the ligand for NOD2 activation, negatively regulates TLR2 signaling. NOD2 deficient mice adoptively transferred with OVA-specific CD4+ T cells, and stimulated with OVA expressing *E. coli* (ECOVA) developed colitis, whereas wild type controls did not. Importantly, this colitis was TLR2 dependent, since inflammation was suppressed in NOD2-TLR2 double deficient mice^[51]. It was later reported that administration of MDP protects mice from the development of experimental colitis by downregulating multiple TLR responses, not just TLR2^[52].

Epidemiological data assessing the role of TLR2 in the pathogenesis of IBD are scarce. None of the nonsynonymous SNPs of TLR1, 2 or 6 were involved with IBD susceptibility. However, a number of variants were found to be associated with disease phenotypes. The TLR2 R753G and TLR1 R80T SNPs were found to be associated with pancolitis in UC. The relative risks for heterozygous patients to develop pancolitis were 5.8 and 3.3 for R80T and R753G, respectively^[53]. There was a negative association between TLR6 S249P SNP and proctitis in UC patients. In CD there was a negative association between ileal disease involvement and TLR1 S602I SNP.

TLR3

This less studied TLR in the pathogenesis of IBD, signals upon activation by double stranded RNA through a Myd88-independent pathway *via* the adaptor TRIF. Double stranded RNA is produced during viral replication as an intermediate of the replication cycle or as part of the viral RNA genome, and is also produced during apoptosis.

In wild type mice, subcutaneous administration of poly (I:C), a synthetic TLR3 agonist, protected against DSS-induced colitis. In contrast, intragastric administration of poly (I:C) offered no protection in this colitis model nor did its administration activate the innate immune system as assessed by serologic parameters^[54].

Activation of TLR3 signaling induced by poly(I:C) was shown to cause an increase in IL-15 secretion leading to mucosal damage in the small intestine. IL-15 is a key regulatory cytokine involved in mucosal homeostasis. IL-15 secretion increased the percentage and number of CD3+NK1.1+ intestinal intraepithelial lymphocytes (IELs) and caused their enhanced cytotoxicity^[55]. TLR3 signaling was also shown to induce IEC expression of

Rae1 (a ligand for NKG2D), which mediates epithelial destruction and mucosal injury by interacting with NKG2D expressed on intestinal intraepithelial lymphocytes^[56].

In humans, TLR3 expression by IECs of UC patients is comparable to that of healthy controls, while TLR3 expression was significantly downregulated in CD patients, both in inflamed and non-inflamed tissue^[20].

CONCLUSION

Inflammatory bowel disease is a chronic relapsing disease of the gastrointestinal tract. Although the etiology is unknown, both innate immunity and the commensal bacterial flora are hypothesized to play a major role in its pathogenesis. The gastrointestinal innate immune system has to recognize, sort and respond to a vast array of microbial products present in the intestinal lumen. TLRs have evolved as the major innate immune surveillance, recognition and response receptors central to efficient host defense and homeostasis of the intestinal mucosa.

The factors directing TLR-regulated immune response in IBD remain poorly understood. TLRs may either enhance or suppress intestinal inflammation. Membrane localization (either basolateral or apical), expression pattern (IECs *versus* regulatory T lymphocytes), parallel signaling by additional TLRs, cytokine combinations and interactions with specific intestinal flora all determine the type and balance of the immune response.

In mouse models, knockout of a single TLR usually does not result in spontaneous colitis. Only after additional genetic and pharmacological interventions does a clear gastrointestinal phenotype emerge. These data suggest that mutations in a single TLR are insufficient to explain the complex pathogenesis of IBD. However, they do suggest that TLRs are crucial for initiation and progression of IBD and play a major role in its pathogenesis.

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TOPIC HIGHLIGHT

Emiko Mizoguchi, MD, PhD, Series Editor

Mucosal cytokine network in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are characterized by ongoing mucosal inflammation in which dysfunction of the host immunologic response against dietary factors and commensal bacteria is involved. The chronic inflammatory process leads to disruption of the epithelial barrier, and the formation of epithelial ulceration. This permits easy access for the luminal microbiota and dietary antigens to cells resident in the lamina propria, and stimulates further pathological immune cell responses. Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial and mesenchymal cells. The clinical efficacy of targeting TNF- α clearly indicates that cytokines are the therapeutic targets in IBD patients. In this manuscript, we focus on the biological activities of recently-reported cytokines [Interleukin (IL)-17 cytokine family, IL-31 and IL-32], which might play a role through interaction with TNF- α in the pathophysiology of IBD.

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INTRODUCTION

Inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are chronic intestinal disorders of unknown etiology^[1,2]. The most widely accepted hypothesis on the pathogenesis of IBD is that the mucosal immune system shows an aberrant response towards luminal antigens such as dietary factors and/or commensal bacteria in genetically susceptible individuals^[1,3,4]. Environmental factors may also precipitate the onset or reactivation of this disease^[1,3,4]. The chronic inflammatory process leads to disruption of the epithelial barrier, and the formation of epithelial ulceration. Abnormal bacterial killing based on genetic factors such as the *NOD2* gene mutation also induces mucosal damage. The easy access for the luminal microbiota and dietary antigens into cells resident in the lamina propria thus stimulates pathological immune cell responses.

Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial and mesenchymal cells^[5]. The clinical efficacy of targeting TNF- α clearly indicates that cytokines are one of the therapeutic targets of chronic inflammatory disorders such as IBD. Infliximab, a mouse/human chimeric mAb that binds to TNF- α , has shown efficacy in the treatment of moderate-to-severe CD and a number of CD-related complications^[6-8]. Although the precise mechanism of the action of infliximab is not clear, it neutralizes soluble TNF- α , alters cell signaling, and induces the apoptosis of activated inflammatory cells expressing surface TNF- α via multiple pathways. On the other hand, some patients are resistant to anti-TNF- α therapy. In such patients, the disease might be driven by molecular mechanisms dependent on other cytokines distinct from TNF- α . In this manuscript, we focused on recently-reported cytokines [Interleukin (IL)-17 cytokine family, IL-31 and IL-32] which might play a role through interactions with TNF- α in the pathophysiology of IBD.

IL-17 CYTOKINE FAMILY

IL-17 family and Th17 cells

The interleukin (IL)-17 cytokine family is a group of T cell derived cytokines. IL-17A was originally cloned by Rouvier *et al.*^[9] and named CTLA8. It was subsequently renamed IL-17, and was more recently termed IL-17A. IL-17A stimulates various cell types to secrete various cytokines and chemokines, resulting in the induction of inflammation^[10-15]. The IL-17 family may play a role in a number of diseases mediated by abnormal immune responses, such as rheumatoid arthritis^[16,17], pulmonary disease^[15,18], lupus^[19], multiple sclerosis^[20], and IBD^[21]. Homology-based cloning recently revealed five additional members of the IL-17 family, termed IL-17B to IL-17F^[10]. Among the IL-17 family members, IL-17F has the highest degree of homology with IL-17A (40% to 55%), followed by IL-17B (29%), IL-17D (25%), IL-17C (23%), and IL-17E (also named IL-25) is the most distantly related (17%)^[10]. The major cellular source of IL-17A was initially described as activated CD4+ memory T lymphocytes. But, it was subsequently demonstrated that CD8+ memory T lymphocytes, eosinophils, neutrophils and monocytes can also produce IL-17A^[10,22]. The cellular sources of IL-17B and IL-17C have not been identified. IL-17D is derived from resting CD4+ T cells and CD19+ B cells^[15]. But, IL-17E (IL-25) expression is restricted to Th2 cells and mast cells^[15]. The cellular source of the last member, IL-17F, has been reported to be activated CD4+ T cells, basophils and mast cells^[15].

One recent topic in immunology is the newly identified Th17 lineage of CD4+ T cells^[23]. Th17 cells are characterized by the production of a distinct profile of effector cytokines, including IL-17A, IL-17F, IL-6, IL-22 and IL-26, and have probably evolved to enhance immune and host defense responses distinct from those targeted by Th1 and Th2 cells^[24-26]. Th17 cells develop from naïve CD4 T cell precursors in the presence of IL-6 and TGF- β , and full differentiation to Th17 cells is dependent on IL-23^[23]. Recent studies demonstrated a role for IL-21 in Th17 development^[24,27]. IL-21 serves as an autocrine factor secreted by Th17 cells that promotes or sustains the Th17 lineage commitment. On the other hand, Th1 cells develop from naïve CD4 T cell precursors in the presence of IFN- γ , whereas Th2 cells develop under the control of IL-4. Both IFN- γ and IL-4 inhibit Th17 cell proliferation^[28]. A recent study showed that the proliferation of Th17 cells is also inhibited by IL-27, an IL-12-related cytokine frequently present at sites of inflammation^[29]. Th17 cells are characterized as a source of IL-17A and IL-17F, and much attention has been focused on their functions in normal and pathological immune responses.

We previously demonstrated that IL-17A-positive cells were increased in the inflamed mucosa of IBD patients^[21], and a recent study showed that IL-17F mRNA expression in the mucosa was elevated in CD patients^[30]. These observations suggest that IL-17F as well as IL-17A might play a role in the inflammatory responses involved in the pathophysiology of IBD.

Table 1 IL-17A-induced genes in human colonic myofibroblasts

Gene name	Ref seq ID.	Fold increase
CXCL1 (Gro- α)	NM_001511.1	× 26.11
IL-20 receptor, alpha	NM_014432.1	× 15.22
CCR5	NM_000579.1	× 12.75
CXCL6 (GCP-2)	NM_002993.1	× 12.45
CXCL3 (Gro- γ)	NM_002090.1	× 11.70
MMP-16	NM_005941.2	× 10.41
CXCL2 (Gro- β)	NM_002089.1	× 9.75
IRAK3	NM_007199.1	× 9.06
IL-8	NM_000584.2	× 8.91
IL-22 receptor 1	NM_021258.1	× 8.22
MMP-1	NM_004142.1	× 6.35
Complement component 3	NM_000064.1	× 5.56
IL-6	NM_000600.1	× 4.63
Carbonic anhydrase XII	NM_001218.1	× 4.42
Superoxide dismutase 2	NM_000636.1	× 4.25
CCL19 (MIP-3 β)	NM_006274.2	× 3.74
CCL7 (MCP-3)	NM_006273.2	× 2.77
CCL2 (MCP-1)	NM_002982.2	× 2.24

Human colonic subepithelial myofibroblasts were stimulated with IL-17A (200 ng/mL) for 12 h, and the changes in gene expression were assessed by IntelliGene HS Human Expression Chip (Takara-Bio, Kyoto, Japan). The fold change values were determined as a ratio of Cy5 signal intensity (IL-17A stimulated values)/Cy3 signal intensity (non-stimulated values). The data were average of three independent analysis. The NCBI reference sequence code was presented following the gene name.

Inflammatory responses induced by IL-17A and IL-17F

IL-17A promotes the expansion and recruitment of innate immune cells such as neutrophils, and also cooperates with TLR ligands, IL-1 β , and TNF- α to enhance inflammatory reactions, and to stimulate the production of beta-defensins and other antimicrobial peptides^[25,27]. Its receptor, IL-17RA, is ubiquitously expressed and shares many features with classical innate immune receptors such as shared intracellular tail motifs and convergence onto common inflammatory transcription pathways^[31]. To investigate the genes altered in response to an IL-17A stimulus, we performed a cDNA microarray analysis in human colonic subepithelial myofibroblasts (SEMFs)^[32]. Human colonic SEMFs are located immediately subjacent to the basement membrane in the normal intestinal mucosa, juxtaposed against the bottom of the epithelial cells^[33,34], and play a role in inflammation and wound healing in the intestine^[33-36]. As shown in Table 1, IL-17A up-regulated several genes which have been reported to exert pro-inflammatory actions in the pathophysiology of acute and/or chronic inflammation. In particular, the induction of CXC-chemokines mRNA expression suggests that IL-17A is a potent inducer of innate immune responses *via* the accumulation and activation of neutrophils in the mucosa.

Recently, we found that among the IL-17 family members, IL-17F also strongly induced the secretion of inflammatory cytokines (IL-6, IL-8 and LIF) and matrix metalloproteinases (MMP-1 and MMP-3) in human colonic SEMFs^[32]. Like IL-17A^[37], IL-17F stimulated IL-6, IL-8 and MCP-1 secretion *via* NF- κ B and MAP kinase activation in human colonic SEMFs. The IL-6,

Table 2 IL-17A augmented TNF- α -induced genes in human colonic myofibroblasts

Gene name	Ref seq ID.	Fold increase
CXCL2 (Gro- β)	NM_00002089.1	\times 34.42
CXCL1 (Gro- α)	NM_001511.1	\times 23.69
IL-6	NM_000600.1	\times 16.92
CXCL3 (Gro- γ)	NM_002090.1	\times 15.20
IL-1 β	NM_000576.2	\times 11.59
GM-CSF	NM_000758.2	\times 9.11
IL-1 α	NM_000575.3	\times 8.45
Amphiregulin	NM_001657.2	\times 6.61
CCL20	NM_004591.1	\times 5.21
MMP-3	NM_002422.2	\times 4.12
CCL7 (MCP-3)	NM_006273.2	\times 3.96
IL-13R α 2	NM_000640.2	\times 3.76
CXCL6 (GCP-2)	NM_002993.1	\times 3.74
IL-11	NM_000641.2	\times 3.70
IL-8 (CXCL8)	NM_000584.2	\times 3.65
PGF2	NM_002006.2	\times 3.12
MMP-1	NM_004142.1	\times 2.49
CCL5 (RANTES)	NM_002985.2	\times 0.43

Human colonic subepithelial myofibroblasts were stimulated with TNF- α (50 ng/mL) or TNF- α (50 ng/mL) plus IL-17A (200 ng/mL) for 12 h, and the changes in gene expression were assessed by IntelliGene HS Human Expression Chip. The fold change values were determined as a ratio of Cy5 signal intensity (TNF- α plus IL-17A stimulated values)/Cy3 signal intensity (TNF- α stimulated values). The data were average of three independent analysis. The NCBI reference sequence code was presented following the gene name.

IL-8 and LIF secretion by human SEMFs in response to IL-17F as well as IL-17A emphasizes the importance of Th17 products in the induction of intestinal inflammation. Furthermore, the induction of IL-6 secretion from colonic SEMFs has a particular significance in the regulation of immune responses, and in the pathophysiology of IBD; IL-6 has recently been identified as an indispensable factor for the development of Th17 cells^[23]. IL-6 commits naïve CD4 T cell precursors to differentiate into Th17 cells^[23]. IL-17A and IL-17F might function as potent stimulators for IL-6 production, suggesting an amplification loop for the local development and maturation of Th17 cells. Therefore, the IL-17-colonic SEMFs-IL-6 axis may be important for local Th17 development in the intestinal mucosa.

IL-17A/IL-17F augments TNF- α -induced inflammatory responses

As more important observations, IL-17A and IL-17F augment the TNF- α -induced IL-6 secretion in human colonic SEMFs^[32,37]. This augmentation of TNF- α -induced IL-6 production by IL-17A is mediated by enhanced stability of the IL-6 mRNA^[37,38]. A similar molecular mechanism can be postulated for the augmentation by IL-17F. IL-17A/IL-17F also augments the TNF- α -induced expression of granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage (GM)-CSF in human colonic SEMFs^[39].

To further investigate the effects of IL-17A/IL-17F on TNF- α -induced genes, the alterations in gene expression were analyzed by cDNA microarrays in hu-

man colonic myofibroblasts. As shown in Table 2, IL-17A further enhanced the expression of various TNF- α -induced genes, such as IL-6, CXC-chemokines and CSFs. These observations suggest that the interactions between TNF- α and IL-17A/IL-17F potentially mobilized neutrophils, partially through granulopoiesis and CXC chemokine induction, as well as through increased survival locally. This interaction also potentially stimulated Th17 development through the stimulation of IL-6 secretion. Thus, a modulation of the immunological functions of colonic SEMFs by Th17-derived cytokines may be critical for the development of Th17 cells and the mucosal innate immune responses (Figure 1).

Interactions between IL-17A and IL-22

IL-22 was originally described as an IL-9-induced gene, and was termed "IL-10-related T cell-derived-inducible factor" (IL-TIF)^[40,41]. IL-22 has 22% amino acid identity with IL-10, and belongs to a family of cytokines with limited homology to IL-10, namely IL-19, IL-20, IL-22, IL-24 and IL-26. The major sources of IL-22 are activated T cells, and IL-22 expression in other leukocyte populations such as monocytes, dendritic cells, NK cells and neutrophils is negligible. Recent studies have shown that Th17 cells are a source of IL-22^[42,43]. We recently found that IL-22 expressing cells were increased in the inflamed mucosa of IBD patients^[44]. In SEMFs, IL-22 upregulates the expression of inflammatory genes such as IL-6, IL-8, IL-11 and LIF *via* NF- κ B, AP-1 and MAP-kinase dependent pathways^[44]. Furthermore, the combination of IL-17A plus IL-22 showed an additive effect on transcription factor activation. These concerted responses were also observed as additive effects on cytokine mRNA expression and protein secretion. Thus, the cooperation between Th17 derived cytokines such as IL-17A and IL-22 may play an important role in the pathophysiology of IBD.

IL-31

IL-31 has a Th2 cell origin

IL-31 was cloned, and then found to be mainly produced by CD4+ T cells^[45], in particular by skin-homing CD45RO+ (memory) T cells. Transgenic mice overexpressing IL-31 either with a lymphocyte-specific promoter or a ubiquitous promoter exhibit a skin phenotype closely resembling atopic dermatitis in human subjects^[45]. In these mice, IL-31 seems to be preferentially produced by T cells skewed towards a Th2 phenotype; however, these Th1-skewed T cells also produce substantial amounts of IL-31^[45]. IL-31 mRNA expression is widely detected in various organs, including the gastrointestinal tract^[45].

IL-31 is most closely related to the family of IL-6-type cytokines known to be involved in many immunomodulatory functions, particularly the acute-phase response, but also in the proliferation of B and T cells^[46]. A recent study indicated that IL-31 sustains the survival of hematopoietic stem cells, and contributes to effects on the cycling and numbers of hematopoietic stem cells

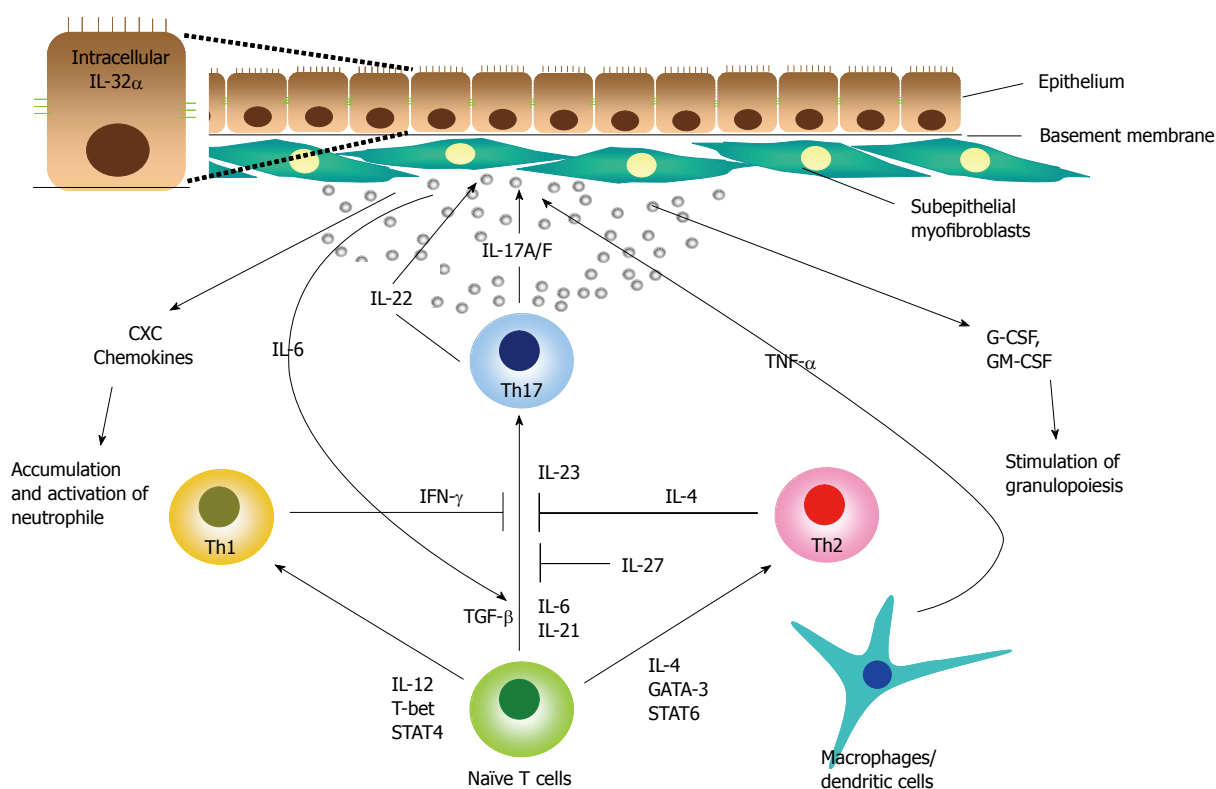


Figure 1 Mucosal cytokine network mediated by IL-17A/F and TNF- α . TGF- β induces the differentiation of Th17 cells from naïve CD4⁺ T cells in the presence of IL-6, and full differentiation to Th17 cells is dependent on IL-23. The transcriptional factors required for the development of Th1 (STAT4 and T-bet) and Th2 (STAT6) cells are not required for the induction of Th17/Th17 cells. IFN- γ also suppresses the differentiation of Th17 cells. IL-4 inhibits the development of Th17 cells. One of the important roles of IL-17A/F is the augmentation of the TNF- α -induced secretion of IL-6, a crucial factor for Th17 cell development.

in vivo^[47]. However, IL-31 is clearly distinct from the IL-6-type cytokines because it does not signal through glycoprotein-130 (GP-130), the common signaling receptor subunit. IL-31 uses an earlier described orphan receptor, the glycoprotein 130-like monocytic receptor or glycoprotein 130-like receptor (GPL)^[48], in combination with the oncostatin M receptor (OSMR)^[45], which is expressed on epithelial cells and keratinocytes^[45,49].

IL-31 binds directly to the GPL, and OSMR mainly plays a role in delivering the signaling information into the cells. GPL and OSMR are widely expressed in various tissues, including the gastrointestinal tract. This suggests a role for IL-31 in the immune and inflammatory responses of the intestine. In response to IL-31, its receptor complex recruits the Jak1, Jak2, STAT-1, STAT-3 and STAT5 signaling pathways, as well as the PI3-kinase/AKT cascade^[49]. SHP-2 and Shc adaptor molecules are also recruited, and contribute to an increased activation of the MAP kinase pathway in response to IL-31^[49]. Despite the extensive study of intracellular signaling pathways activated by IL-31 stimulation, the cellular responses to IL-31 were barely investigated in any cell type.

IL-31 stimulates inflammatory responses in colon myofibroblasts

To define the role of IL-31 in the intestinal mucosa, we investigated how IL-31 modulates mRNA expression in human colonic SEMFs. An analysis of the cDNA

microarrays indicated that IL-31 effectively induced the secretion of chemokines [CXCL8 (IL-8), CXCL1 (growth-related oncogene; GRO- α), CCL7 (monocyte chemoattractant protein-3; MCP-3), CXCL3, CCL13, CCL15], proinflammatory cytokines (IL-6, IL-16 and IL-32), and matrix metalloproteinases (MMP-1, MMP-3, MMP-25 and MMP-7). The stimulatory effects of IL-31 were comparable to the effects of IL-17A. Furthermore, simultaneous stimulation with IL-31 and IL-17A showed additive effects on IL-6, IL-8, GRO- α , MCP-3, MMP-1 and MMP-3 secretion. Similar effects for IL-31 have been reported in bronchial epithelial cells^[50]. In bronchial epithelial cells, IL-31 could significantly elevate both gene and protein expressions of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1/CCL2). The combination of IL-31 with either IL-4 or IL-13 further enhanced VEGF and CCL2 production. In these cells, IL-31 could activate p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK).

As mentioned above, IL-31 is a Th2 type cytokine, and these findings indicate that Th2 cells may be involved in the immune and inflammatory responses of the intestinal mucosa through IL-31 secretion. Since IL-31 and IL-17A stimulate the secretion of proinflammatory mediators in an additive manner, Th2-derived IL-31 and Th17-derived IL-17A cooperate in the pathophysiology of IBD.

IL-32

IL-32 cytokine family

IL-32 is a recently described cytokine produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells^[51,52]. IL-32 is a proinflammatory cytokine originally described as a transcript termed NK4, found in activated natural killer (NK) cells and T lymphocytes^[53]. Although IL-32 was first reported as a transcript in IL-2 activated NK and T cells, it appears that the epithelial cells are the dominant and widespread source^[54]. The gene encoding IL-32 is located on human chromosome 16p13.3, and is organized into eight exons^[55]. There are four splice variants (IL-32 α , IL-32 β , IL-32 δ and IL-32 γ), and IL-32 α is the most abundant transcript. Of particular importance, IL-32 is prominently induced by interferon (IFN)- γ in lung epithelial cells and monocytes^[51]. IL-32 stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1 β , TNF- α , IL-6 and IL-8 by *via* the activation of NF- κ B and p38 mitogen-activated protein kinases (MAPKs)^[51,52]. IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis^[54,56-58], mycobacterium tuberculosis infection^[59,60], and IBD^[61].

Intracellular accumulation of IL-32

The amino acid sequence derived from the initial NK4 cDNA contained a signal peptide without a transmembrane domain^[53,54]. But, the transcript was never expressed as a recombinant protein, and was not sequenced. Activated human T cells generate IL-32 with a molecular weight of 25 kDa, which on Western blotting analysis is found in the lysates rather than the supernatants. Similar findings were reported for 293T cells transfected with either IL-32 γ or IL-32 β ^[62]. In human peripheral blood mononuclear cells stimulated with ConA, most of the IL-32 was found in the lysates^[51]. On the other hand, the overexpression of IL-32 α or IL-32 β in COS cells resulted in secreted IL-32^[51]. It remains unclear which isoforms are secreted from which particular cell type. Activated T cells and NK cells do not secrete IL-32, or alternatively, the secreted IL-32 is derived from apoptotic cells due to the presence of GAPDH in the same supernatants. These observations suggest that IL-32 is secreted only as a result of cell death^[63]. Recently, we observed that in colon cancer cell lines, proinflammatory cytokines induce the intracellular accumulation of IL-32 α , but does not induce secretion^[61]. Similar results were also observed in myofibroblasts isolated from the normal human pancreas^[64]. Goda *et al* suggest that there is a role for intracellular IL-32 with cell death, since attenuating intracellular IL-32 levels resulted in decreased cell death^[62]. These results also support the concept that high levels of intracellular IL-32 β may induce cell death. One hypothesis is that the proinflammatory activity of IL-32 may act upon its release through cell death (apoptosis).

Molecular mechanisms regulating IL-32 α induction

Shioya *et al* demonstrated that stimulation with IL-1 β ,

IFN- γ and TNF- α enhanced IL-32 α mRNA expression in three colon cancer cell lines^[61]. TNF- α was the strongest among them. These factors also induced the intracellular accumulation of IL-32 α . Since transfection with the mutant form of I κ B α inhibited the effects of both IL-1 β and TNF- α on IL-32 α mRNA expression, NF- κ B must play a role in IL-1 β - and TNF- α -induced IL-32 α mRNA expression.

Nishida *et al* analyzed IL-32 α expression in non-transformed myofibroblasts derived from the normal human pancreas^[64]. IL-32 α mRNA was weakly expressed without any stimulus, and its expression was markedly enhanced by IL-1 β , IFN- γ and TNF- α . IL-1 β , IFN- γ and TNF- α enhanced the intracellular accumulation of IL-32 α protein. But, IL-32 α was not detected in the supernatants. An inhibitor of phosphatidylinositol 3-kinase, (LY294002) significantly suppressed the IL-1 β -, IFN- γ - and TNF- α -induced IL-32 α mRNA expression, although MAPK inhibitors had no effect. Akt activation in response to these cytokines was confirmed by Western blotting analysis. Furthermore, LY294002 suppressed both IL-1 β - and TNF- α -induced NF- κ B activation, as well as IL-1 β -, TNF- α - and IFN- γ -induced AP-1 activation. A blockade of NF- κ B and AP-1 activation by an adenovirus expressing a stable mutant form of I κ B α and a dominant negative mutant of c-Jun markedly suppressed the IL-1 β -, IFN- γ - and/or TNF- α -induced IL-32 α mRNA expression. Thus, they concluded that IL-32 α mRNA expression was dependent on interactions between the PI3K/Akt-pathway and the NF- κ B/AP-1 system.

IL-32 α and IBD

Shioya *et al* performed an immunohistochemical analysis to evaluate the expression of IL-32 α protein in the mucosa of IBD patients^[61]. IL-32 α was weakly immunexpressed by epithelial cells in the normal colonic mucosa and samples of ischemic colitis. In contrast, the epithelial expression of IL-32 α was markedly enhanced in the inflamed region of active UC and CD patients^[61]. In particular, IL-32 α expression tends to increase in samples from active CD patients. IL-32 α expression was barely detectable in leukocytes. Thus, these observations indicate that epithelial cells are the major expression site for IL-32 α in the intestinal mucosa, and that IL-32 α expression is enhanced in the IBD mucosa.

Netea *et al* recently demonstrated that IL-32 augments the production of IL-1 β and IL-6 induced by muramyl dipeptide (MDP), a peptidoglycan fraction of bacteria, by means of nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism^[52]. NODs are a family of intracytoplasmic bacterial sensors, and the recognition of bacterial peptidoglycans subsequently induces NF- κ B activation^[65]. Mutations in NOD2 have been implicated in the pathogenesis of CD^[66,67], and CD patients homozygous for the frameshift 3020insC mutated allele have defective responses to MDP in terms of cytokine production^[68,69]. Recently, it has been shown that NOD2 mutations in CD patients potentiate NF- κ B activity

and IL-1 β processing^[70]. Thus, these findings suggest a pivotal role for IL-32 in the pathophysiology of IBD, and in particular CD. Since IL-32 α is a proinflammatory cytokine characterized by NF- κ B and p38 MAPK activating activities^[51,57] and because IL-32 acts synergistically with NOD ligands to induce proinflammatory cytokines^[52], the overexpression of IL-32 α in the IBD mucosa strongly suggests that it plays an important role in the inflammatory and antibacterial responses involved in the pathogenesis of IBD.

Recent studies have focused on the role of innate immunity in the pathogenesis of IBD^[4]. The initial step of innate immunity is mediated by the recognition of pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs) and NOD proteins (NODs)^[71]. TLRs are located mainly on cell-surface membranes, but NODs function as intracellular recognition systems^[65,66]. In human monocytes, IL-32 acts synergistically with NOD specific peptidoglycans for the release of IL-1 β and IL-6^[52]. The synergistic effects of IL-32 and the NOD ligands on cytokine production is abolished in cells from CD patients bearing the NOD2 frameshift mutation 3020insC, indicating that this synergism between IL-32 plus MDP depends on NOD2^[52]. Interactions between NOD-1 and IL-32 also potentiate proinflammatory cytokine production^[52]. Furthermore, Berrebi *et al* previously reported the overexpression of NOD2 in infiltrated monocytes and epithelial cells in the IBD mucosa^[72]. These observations suggest that overexpressed IL-32 may cause a specific and excessive stimulation of the NOD pathways, which leads to a marked amplification in IL-1 β and IL-6 production in the IBD mucosa.

IL-32 was initially characterized as an inducer of TNF- α in circulating monocytes^[51], and hence inflammatory responses in the affected mucosa of IBD patients may be amplified by the consecutive loop of IL-32-induced TNF- α secretion from monocytes and TNF- α -stimulated IL-32 secretion from epithelial cells. This loop may be further amplified by the Th1 cytokine IFN- γ . Previously, it has been reported that TNF- α and IFN- γ synergistically induced the release of NOD2^[73], which supports the coupled regulation of IL-32 α and NOD2. The coupled regulation of IL-32 α with NOD2 may account for the rapid and efficient induction of innate immune responses at the intestinal mucosa. Furthermore, these data suggest that an amelioration of IBD symptoms by TNF- α -targeting therapies may be partially dependent on interference in the TNF- α -IL-32 loop.

The apoptosis of IECs is considered a normal biological function to eliminate damaged epithelial cells, and to restore epithelial cell growth, regulation, and epithelial integrity^[74]. An overexpression of cytoplasmic IL-32 α might account for the induction of apoptosis in damaged epithelial cells at the inflamed mucosa of IBD patients, leading to an efficient elimination and the rapid induction of mucosal repair. Apoptosis caused by accumulated IL-32 can be considered a host defense mechanism against invading microorganisms, in which

damaged epithelial cells are efficiently eliminated along with the invading microorganisms, and thus any further invasion of the microorganisms can be blocked.

CONCLUSION

In this review, we have summarized the newly reported cytokines which may play significant roles in the pathophysiology of IBD. An augmentation of TNF- α effects by IL-17A/F and a possible amplifying cascade between TNF- α and epithelial-derived IL-32 are of particular interest. The clinical efficacy of TNF- α blocking may be associated with an interruption of these cascades. The discovery of new cytokines and the determination of their biological activities may support the development of a novel therapeutic strategy for the treatment of IBD patients.

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COLORECTAL CANCER

Roscovetine synergizes with conventional chemo-therapeutic drugs to induce efficient apoptosis of human colorectal cancer cells

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Abstract

AIM: To examine the ability of cyclin-dependent kinase inhibitor (CDKI) roscovetine (Rosco) to enhance the antitumor effects of conventional chemotherapeutic agents acting by different mechanisms against human colorectal cancer.

METHODS: Human colorectal cancer cells were treated, individually and in combination, with Rosco, taxol, 5-Fluorouracil (5-FU), doxorubicin or vinblastine. The antiproliferative effects and the type of interaction of Rosco with tested chemotherapeutic drugs were determined. Cell cycle alterations were investigated by fluorescence-activated cell sorter FACS analysis. Apoptosis was determined by DNA fragmentation assay.

RESULTS: Rosco inhibited the proliferation of tumor cells in a time- and dose-dependent manner. The efficacies of all tested chemotherapeutic drugs were markedly enhanced $3.0-8.42 \times 10^3$ and $130-5.28 \times 10^3$ fold in combination with 5 and 10 $\mu\text{g/mL}$ Rosco, respectively. The combination of Rosco and chemotherapeutic drugs inhibited the growth of human colorectal cancer cells in an additive or synergistic fashion, and in a time and dose dependent manner. Rosco induced apoptosis and synergized with tested chemothera-

peutic drugs to induce efficient apoptosis in human colorectal cancer cells. Sequential, inverted sequential and simultaneous treatment of cancer cells with combinations of chemotherapeutic drugs and Rosco arrested the growth of human colorectal cancer cells at various phases of the cell cycle as follows: Taxol/Rosco (G_2/M - and S-phases), 5-FU/Rosco (S-phase), Dox/Rosco (S-phase) and Vinb/Rosco (G_2/M - and S-phases).

CONCLUSION: Since the efficacy of many anticancer drugs depends on their ability to induce apoptotic cell death, modulation of this parameter by cell cycle inhibitors may provide a novel chemo-preventive and chemotherapeutic strategy for human colorectal cancer.

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Key words: Human colorectal cancer cell lines; Cyclin dependent kinase inhibition; Chemosensitization; Synergy; Apoptosis; Cell cycle

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INTRODUCTION

Colon cancer is the second leading cause of cancer death in the United States and is one of the most common cancers in Western countries^[1]. Lack of improvement in overall survival and failure of the current systemic therapies have mandated that new approaches to this disease be explored.

A hallmark of neoplastic evolution and progression is deregulation of cell cycle control mechanisms. The key regulators of transition from one cell cycle phase to the next are the cyclin-dependent kinases (CDKs).

CDKs are serine/threonine kinases that regulate cell cycle progression in a highly coordinated manner^[2]. A CDK enzyme complex becomes fully active after binding of its proper cyclin. Progression through cell cycle is mediated by the orchestrated activation and breakdown of CDK complexes^[2].

A basis for selectivity of CDK-directed therapies against neoplastic cells might arise from the fact that alteration of CDK structure and function plays a key role in the pathogenesis of neoplasia^[3]. At least one of the following changes is almost ubiquitously evident in human neoplasms: overexpression of cyclin D; amplification or structural alteration of CDK4; deletion or mutation of p16^{INK4A}; mutation of the CDK4 or 6/cyclin D substrate p^{Rb}; and loss of p21^{WAF1/CIP1} function through deletion or mutation of its transactivator P53. In relation to colon cancer, p27^{KIP1} loss has been found to occur not by gene deletion or mutation, but by increased proteolysis of the CDK inhibitor (CDKI)^[4]. Therefore, replacement of at least some of the missing capacity to inhibit cell cycle progression may restore some measure of cell cycle control. In contrast to their normal counterparts, transformed cells proliferate very rapidly due to the enhanced activity of the CDK^[2]. Thus, inhibition of CDK/cyclin complexes offers a promising therapeutic strategy in the defense against cancer^[5].

Many types of potential CDK modulators are conceivable. These include molecules that directly inhibit ATP or protein substrate binding; alter regulatory phosphorylations of the catalytic subunit; inhibit CDK catalytic subunit binding with its respective cyclin or other accessory proteins; mimic the action or increase the expression of endogenous CDK inhibitors, p16^{INK4A} (or its homologues, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D}), p21^{WAF1}, and p27^{KIP1}; interfere with the proper appearance and disappearance of cyclins; and finally alter normal signals for import of CDKs into the nucleus or localization to appropriate subcellular structures^[6].

Pharmacological inhibitors of CDKs display selective anti-proliferative effects on cycling cells, especially malignant ones^[7]. Depending on the selectivity profile of these novel drugs, growth inhibition in different phases of the cell cycle is observed^[8]. Compounds targeting the activity of CDK4/6 block cells in early G₁, whereas selective inhibitors of CDK1/2 arrest cell cycle in G₁/S and G₂/M^[8]. Interestingly, some inhibitors, especially those targeting the activity of CDK2, are able to selectively induce apoptosis in cancer cells^[9,10].

CDKIs, representing a well-defined group of biologically active compounds, are structurally related to adenosine-5-triphosphate, ATP^[7,8,11]. They antagonize binding of kinases to ATP. Differentially substituted adenines yielded a group of inhibitors such as roscovitine (Rosco), olomoucine and purvalanol^[7]. These close analogs, characterized by increasing potency, differ in selectivity. Due to their selectivity and relative low direct cytotoxicity, CDKIs clearly provide useful anticancer drugs and offer an alternative to classic chemotherapeutics. In the present study, we have investigated whether Rosco could inhibit the growth of human colorectal

cancer cells and increase their sensitivity to conventional chemotherapies.

MATERIALS AND METHODS

Cell culture and reagents

Human colorectal cancer cell lines (SW48, SW1116 and SW 837) were obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA). Cells were cultured in Leibovitz's L-15 medium supplemented with 10% inactivated fetal bovine serum and 2 mmol/L glutamine. The L-15 medium formulation was devised for use in a free gas exchange with atmospheric air. CO₂ is detrimental to cells when using this medium for cultivation. All the other chemicals were purchased from Sigma Chemical Co.

Time and dose dependency of the antiproliferative effects induced in human colorectal cancer cells by treatment with Rosco

Human colorectal cancer cell lines (SW48, SW1116 and SW837) were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non-CO₂ incubator. Cells were treated with various concentrations of Rosco (0-40 µg/mL) or DMSO (0.3% final concentration) for various time periods beginning at 24 h after seeding the cells in culture. Control cells were untreated. Cell proliferation was determined at various time intervals (24-168 h) by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described^[12].

In vitro efficacy of single and combined treatment of Rosco and chemotherapeutic drugs on the growth of human colorectal cancer cells

Human colorectal cancer cell lines (SW48, SW1116 and SW837) were treated with taxol (10^{-11} - 10^{-6} mol/L), doxorubicin (10^{-11} - 10^{-6} mol/L), 5-fluorouracil (5-FU) (10^{-9} - 10^{-4} mol/L), vinblastine (10^{-12} - 10^{-7} mol/L) or combinations of the tested chemotherapeutic drug and Rosco (5 and 10 µg/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using the MTT assay.

Analysis of the type of interaction between Rosco and chemotherapeutic drugs in human colorectal cancer cells

Human colorectal cancer cell lines (SW48, SW1116 and SW837) were treated with Rosco (1, 10, 15, 20, 25 µg/mL) and chemotherapeutic drugs (0.5 IC₅₀-4 IC₅₀) individually and in combination. The effect of the combinations of Rosco and chemotherapeutic drugs on cell growth was determined as previously described^[12,13] using the following formulae: $SF_{A+B} > (SF_A) \times (SF_B)$, antagonistic; $SF_{A+B} = (SF_A) \times (SF_B)$; additive; $SF_{A+B} < (SF_A) \times (SF_B)$, synergistic, where SF is the surviving fraction, and A and B indicate the agents used alone, while A + B refers to the agents used in combination.

Cell cycle analysis

Cell cycle phase distribution of human colorectal cancer

Table 1 IC₅₀ and sensitization ratio of taxol and its combinations with Rosco towards human colorectal cancer cell lines

Treatment with taxol and combinations with Rosco	IC ₅₀ (mol/L)			Sensitization ratio ¹		
	SW48	SW1116	SW837	SW48	SW1116	SW837
Taxol	4.8×10^{-8}	1.2×10^{-7}	3.8×10^{-7}	-	-	-
Taxol + 5 µg/mL Rosco	7.4×10^{-10}	1.0×10^{-8}	5.4×10^{-8}	65	12	7
Taxol + 10 µg/mL Rosco	7.5×10^{-11}	8.1×10^{-10}	6.7×10^{-10}	6.4×10^2	1.5×10^2	5.7×10^2

¹Sensitization ratio = IC₅₀ (taxol)/IC₅₀ (taxol + Rosco).

cells treated with Rosco, chemotherapeutic drugs and their combinations was determined by flow cytometry as previously described^[12]. Human colorectal cancer cells (SW837, 5×10^5 cells/well in 24 well plates) were treated with taxol (12×10^{-8} mol/L, 72 h), doxorubicin (8×10^{-7} mol/L, 72 h), 5-FU (4.8×10^{-5} mol/L, 72 h), vinblastine (2.6×10^{-7} mol/L, 72 h), Rosco (15 µg/mL) and combinations of Rosco and chemotherapeutic drugs. The combinations were added in a sequential manner, drug (24 h) followed by Rosco (48 h); inverted sequential manner, Rosco (24 h) followed by drug (48 h) and simultaneous manner (72 h). The tested cells were collected by trypsinization, and then washed with cold phosphate-buffered saline, and counted using a cell counter. A sample of 3×10^6 cells/mL was processed using DNA-Prep kit (Beckman & Coulter, Fa., USA) and a DNA-Prep Epics workstation (Beckman & Coulter). During this process, the cell sample was treated with a cell membrane-permeabilizing agent and then with propidium and RNase enzyme. The sample was then incubated at room temperature for at least 15 min before analysis by aligned flow cytometry (Epics XL, Beckman & Coulter). The percentage of cells in different cell cycle phases was evaluated using the Phoenix statistical software package, advanced DNA cell cycle software (Phoenix Flow System, San Diego, Calif, USA).

DNA fragmentation analysis

DNA fragmentation assay was performed as previously described^[14]. Briefly, colorectal cancer cells (5×10^5 cells/well, SW1116 and SW837) were treated with taxol (1.2×10^{-7} mol/L), doxorubicin (8×10^{-7} mol/L), 5-FU (4.8×10^{-5} mol/L), vinblastine (2.6×10^{-7} mol/L), Rosco (15 µg/mL) and the combinations of Rosco, and tested drugs for 72 h. The cell pellets were lysed with 100 µmol/L of hypotonic buffer (10 mmol/L Tris (pH 8.0), 20 mmol/L EDTA containing 0.5 % Triton X-100) for 30 min at 4°C. Following cell lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 12000 g. The supernatants containing DNA were precipitated overnight with 0.5 mol/L NaCl and 50% isopropyl alcohol at -20°C. Pellets were recovered by centrifugation at 12000 g for 10 min, air dried, resuspended in 30 µL of TE buffer supplemented with 1 mg/mL RNase I at 37°C for 30 min, and then with 2 mg/mL of proteinase K for another 1 h. DNA samples were supplemented with 3 µL of sample buffer (0.25% bromophenol blue, 30% glyceric acid), and elec-

trophoretically separated on a 1.5% agarose gel containing 0.1 µg/mL ethidium bromide at 80 V for 2 h. DNA fragments were visualized by ultraviolet transillumination.

Statistical analysis

Results are representative of two to three individual experiments. Errors are expressed as standard errors of the percentage of the means. Where appropriate, data were analyzed using ANOVA.

RESULTS

Inhibition of the proliferation of human colorectal cancer cells by Rosco

In this study, we initially investigated the effect of Rosco on the proliferation of human colorectal cancer cells using three human colorectal cancer cell lines (SW48, SW1116 and SW837). The results, shown in Figure 1, indicated that all cell lines tested were sensitive to micromolar range of Rosco, in a dose and time dependent manner. Rosco inhibited the growth of SW48 after 24-144 h of drug treatment. It affected the growth of the colorectal cancer cell line SW1116 slightly after 48 h of treatment. However, a dramatic inhibition of cell growth was observed after 72-144 h of treatment. Rosco slightly affected the growth of SW837 after 24 h of treatment. However, a marked inhibition was observed after 48-144 h of treatment with Rosco.

Modulation of taxol cytotoxicity on human colorectal cancer cells by combination with CDKI Rosco

The ability of Rosco to enhance the sensitivity of human colorectal cancer cells to taxol was assessed by treating human colorectal cancer cell lines SW48, SW1116 and SW837 with taxol (10^{-11} - 10^{-6} mol/L) or taxol (10^{-11} - 10^{-6} mol/L) and Rosco (5 or 10 µg/mL) for 96 h. The results summarized in Figure 2A and Table 1 clearly indicated that the combination of taxol and 5 µg/mL Rosco enhanced (65-fold) the anticancer activity of taxol on human colorectal cancer cell line SW48 (Table 1). The difference between SW48 growth inhibition produced by treatment with taxol alone [IC₅₀ (taxol) = 4.8×10^{-8} mol/L] and that produced by treatment with the combination of taxol and 5 µg/mL Rosco [IC₅₀ (taxol + 5 µg/mL Rosco) = 7.4×10^{-10} mol/L] (Table 1) was statistically non-significant ($P = 0.127$). The combination of taxol and 10 µg/mL Rosco greatly increased the sensitivity of human colorectal cancer cells to taxol (640-fold) compared to treatment with

Table 2 Analysis of the combined effects of taxol and Rosco on human colorectal cancer cell lines

Combined treatment of taxol and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells ¹								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
Taxol 0.5 IC ₅₀ + Rosco 1.0 µg/mL	ant	ant	ant	ant	ant	syn	ant	ant	syn
Taxol 1.0 IC ₅₀ + Rosco 10 µg/mL	ant	ant	add	ant	ant	ant	ant	ant	syn
Taxol 2.0 IC ₅₀ + Rosco 15 µg/mL	ant	ant	ant	syn	ant	ant	ant	ant	ant
Taxol 3.0 IC ₅₀ + Rosco 20 µg/mL	ant	ant	ant	ant	ant	ant	ant	ant	ant
Taxol 4.0 IC ₅₀ + Rosco 25 µg/mL	ant	add	add	syn	ant	add	ant	ant	ant

¹The data are based on the mean of absorbance measurements of 3 independent experiments. ant: Antagonistic; add: Additive; syn: Synergistic.

taxol alone (Table 1). The difference in SW48 growth inhibition produced by treatment with combination of taxol and 10 µg/mL Rosco (IC₅₀ = 7.5×10^{-11} mol/L) and that produced by treatment with taxol alone (IC₅₀ = 4.8×10^{-8} mol/L) (Table 1) was significant ($P = 0.012$).

The combination of taxol and 5 µg/mL Rosco had a higher growth inhibitory effect on SW1116 (12-fold) compared to treatment with taxol alone (Table 1). The difference in SW1116 growth inhibition produced by treatment with the combination of taxol and 5 µg/mL Rosco (IC₅₀ = 1×10^{-8} mol/L), and that produced by treatment with taxol alone (1.2×10^{-7} mol/L) was non-significant ($P = 0.256$). On the other hand, the combination of taxol and 10 µg/mL Rosco markedly increased SW1116 growth inhibition (150-fold) compared to treatment with taxol alone (Table 1). The difference in SW1116 growth inhibition produced by the combination of taxol and 10 µg/mL Rosco (IC₅₀ = 8.1×10^{-10} mol/L) and that produced by taxol alone (1.2×10^{-7} mol/L) was statistically significant ($P = 0.03$).

Treatment of SW837 cells with various concentrations of taxol inhibited their growth in a dose dependent manner with IC₅₀ = 3.8×10^{-7} mol/L. The combination of taxol and 5 µg/mL Rosco produced higher SW837 growth inhibition (IC₅₀ = 5.4×10^{-8} mol/L, sensitization ratio = 7-fold) than that produced by treatment with taxol alone (Table 1). This difference in SW837 growth inhibition was non-significant ($P = 0.365$). Treatment of SW837 with the combination of taxol and 10 µg/mL Rosco resulted in a more significant growth inhibition (IC₅₀ = 6.7×10^{-10} mol/L, sensitization ratio = 570 and $P = 0.045$) compared to that produced by treatment with taxol alone (Figure 2, Table 1). The combinations of taxol and Rosco produced additive and/or synergistic effects depending upon the type of cell line used, relative concentrations of the mixed drugs and exposure time (Table 2).

Cell cycle analysis of human colorectal cancer cells treated with taxol, Rosco or their combinations added in sequential (taxol followed by Rosco), inverted sequential or simultaneous manner was determined by flow cytometry as described in Materials and Methods. Treatment of colorectal cancer cells with taxol or Rosco resulted in growth arrest at G₂/M phase, 24.9% and 16.6%, respectively, compared to 4.75% for untreated cells (Figure 1B). The combination of taxol and Rosco added in sequential manner growth arrested colorectal cancer cell in G₂/M (21.8%). Also, the same combination added in

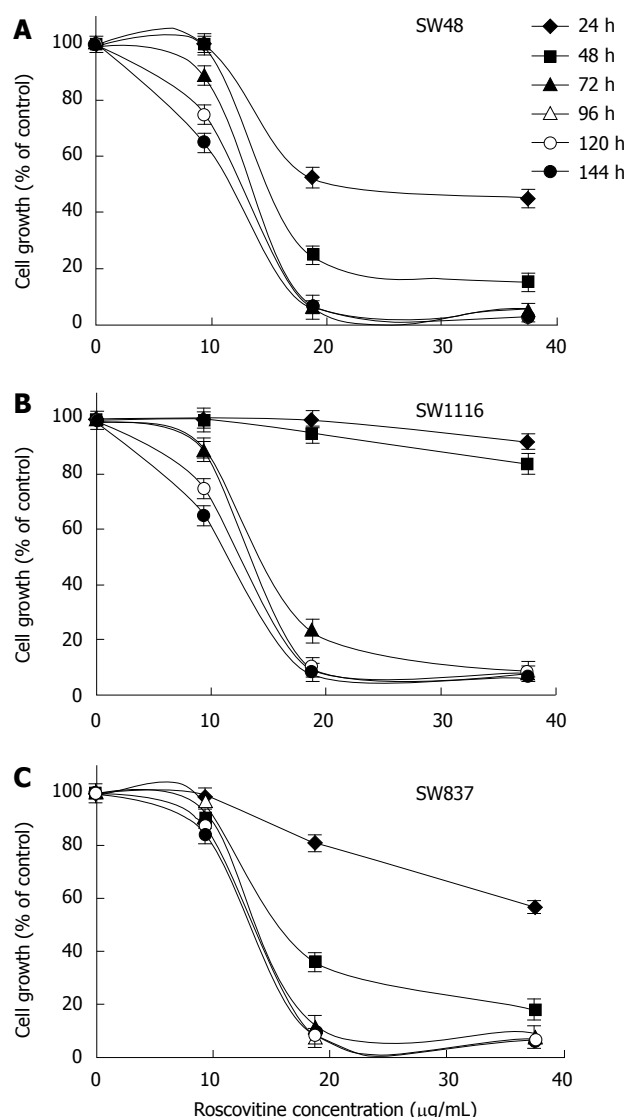


Figure 1 Time and dose dependent effect of roscovitine on the proliferation of human colorectal cancer cell lines. Human colorectal cancer cell lines SW48 (A), SW1116 (B) and SW837 (C) were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non-CO₂ incubator. Twenty four hours after starting the culture, the cells were treated with various concentrations of Rosco (0–40 µg/mL) or DMSO (0.3%, final concentration) for various time periods (24–144 h). Control (0.3% DMSO treated) and Rosco treated colorectal cancer cells were scored for proliferation using an MTT assay. Roscovitine concentrations 5 µg/mL and 10 µg/mL were used in the subsequent studies.

an inverted sequential manner growth arrested colorectal cancer cells in G₂/M (23.4%), meanwhile when the

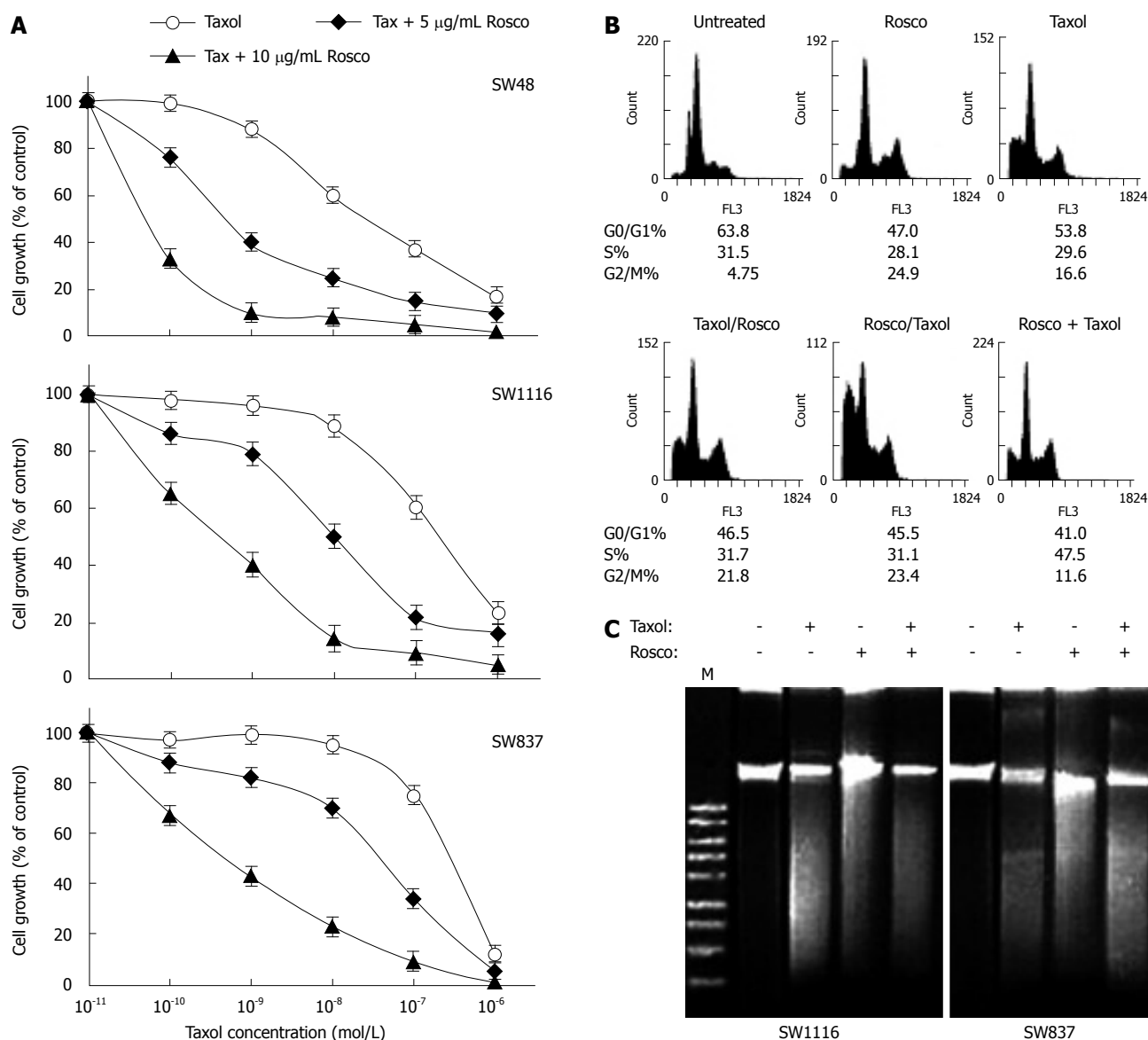


Figure 2 Potentiation of anticancer effect of taxol on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A:** Human colorectal cancer cell lines were treated with taxol (10^{-11} - 10^{-6} mol/L), or the combination of taxol (10^{-11} - 10^{-6} mol/L) and Rosco (5 μg/mL or 10 μg/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using the MTT assay; **B:** Cell cycle phase distribution of human colorectal cancer cells (SW837, 5×10^5 cells/well) treated with taxol (12×10^{-8} mol/L, 72 h); Rosco (15 μg/mL, 72 h); sequential combination: taxol (12×10^{-8} mol/L, 24 h) followed by Rosco (15 μg/mL, 48 h); inverted sequential combination: Rosco (15 μg/mL, 24 h) followed by taxol (12×10^{-8} mol/L, 48 h) and simultaneous combination: taxol plus Rosco (12×10^{-8} mol/L, 15 μg/mL, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated by cell cycle analysis software, Multicycle (Phoenix Flow System, San Diego CA, USA); **C:** Human colorectal cancer cells, SW1116 and SW837 (5×10^5 cells/well), were treated with taxol (12×10^{-8} mol/L), Rosco (15 μg/mL), and the combination of taxol plus Rosco (12×10^{-8} mol/L + 15 μg/mL) for 72 h. DNA fragments were extracted and analyzed on 1% agarose gel.

same combination was used in a simultaneous manner, colorectal cancer cells were growth arrested in G₂/M (11.6%) and S (47.5%) phases, respectively, (Figure 2B). The effect of taxol, Rosco and their combination on inducing programmed cell death in human colorectal cancer cell lines SW1116 and SW837 was studied using DNA fragmentation analysis. The combination of taxol and Rosco induced apoptosis more efficiently compared to single treatment with taxol or Rosco (Figure 2C).

Modulation of 5-FU cytotoxicity on human colorectal cancer cells by combination with the CDKI Rosco

The anti-proliferative activities of 5-FU, Rosco and their

combinations against human colorectal cancer cells are summarized in Figure 3. The combination of 5-FU (10^{-9} - 10^{-4} mol/L) and Rosco (5 μg/mL) exerted a very potent anticancer effect ($P < 0.0001$) on SW48 with $IC_{50} = 5.7 \times 10^{-9}$ mol/L compared to $IC_{50} = 4.8 \times 10^{-5}$ mol/L when SW48 cells were treated with 5-FU alone (Figure 3A). These results indicate that Rosco (5 μg/mL) increased the sensitivity of SW48 to 5-FU by about 8.42×10^3 -fold (Table 3). The same combination inhibited the growth of colorectal cancer cell lines SW1116 and SW837 with IC_{50} values equal to 6.7×10^{-6} mol/L and 1.8×10^{-6} mol/L compared to IC_{50} values equal to 2.5×10^{-5} mol/L and 4.8×10^{-5} mol/L obtained when these

Table 3 IC₅₀ and sensitization ratio of 5-FU and its combinations with Rosco towards human colorectal cancer cell lines

Treatment with 5-FU and combinations with Rosco	IC ₅₀ (mol/L)			Sensitization ratio ¹		
	SW48	SW1116	SW837	SW48	SW1116	SW837
5-FU	4.8 × 10 ⁻⁵	2.5 × 10 ⁻⁵	4.8 × 10 ⁻⁵	-	-	-
5-FU + 5 µg/mL Rosco	5.7 × 10 ⁻⁹	6.7 × 10 ⁻⁶	1.8 × 10 ⁻⁶	8.42 × 10 ³	3.73	26.7
5-FU + 10 µg/mL Rosco	5.7 × 10 ⁻⁹	1.0 × 10 ⁻⁷	9.1 × 10 ⁻⁹	8.42 × 10 ³	250	5.28 × 10 ³

¹Sensitization ratio = IC₅₀ (5-FU)/IC₅₀ (5-FU + Rosco).

Table 4 Analysis of the combined effects of 5-FU and Rosco on human colorectal cancer cell lines

Combined treatment of 5-FU and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells ¹								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
5-FU 0.5 IC ₅₀ + Rosco 1.0 µg/mL	ant	ant	ant	syn	syn	syn	ant	ant	add
5-FU 1.0 IC ₅₀ + Rosco 15 µg/mL	ant	ant	ant	syn	syn	syn	ant	syn	add
5-FU 2.0 IC ₅₀ + Rosco 20 µg/mL	ant	ant	ant	syn	add	add	add	ant	ant
5-FU 3.0 IC ₅₀ + Rosco 25 µg/mL	ant	ant	add	ant	ant	ant	add	ant	ant

¹The data are based on the mean of absorbance measurements of 3 independent experiments; ant: Antagonistic; add: Additive; syn: Synergistic.

cells were treated with Rosco alone, respectively, (Figure 3A, Table 3). Rosco (5 µg/mL) increased the sensitivity of SW1116 and SW837 to 5-FU by about 3.73 and 26.7 fold, respectively. The increase in SW1116 and SW837 growth inhibition after treatment with this combination was found to be statistically non-significant $P = 0.519$ and $P = 0.122$, respectively, compared to single treatment with 5-FU.

The combination of 5-FU (10⁻⁹-10⁻⁴ mol/L) and Rosco (10 µg/mL) exerted a marked growth inhibition on all the tested colorectal cancer cells SW48 ($P < 0.0001$), SW1116 ($P = 0.05$) and SW837 ($P = 0.005$) with IC₅₀ values equal to 5.7 × 10⁻⁹ mol/L, 1.0 × 10⁻⁷ mol/L and 9.1 × 10⁻⁹ mol/L, respectively, compared to IC₅₀ values of 4.8 × 10⁻⁵ mol/L, 2.5 × 10⁻⁵ mol/L, and 4.8 × 10⁻⁵ mol/L exerted on SW 48, SW1116 and SW837, respectively, when treated with 5-FU alone (Figure 3A, Table 3). The combination of 5-FU (10⁻⁹-10⁻⁴ mol/L) and Rosco (10 µg/mL) increased the sensitivity of human colorectal cancer cell lines SW48, SW1116 and SW837 by 8.42 × 10³, 250 and 5.28 × 10³ fold, respectively (Table 3). The combination of 5-FU and Rosco had synergistic effects on SW1116, additive effects on SW837 and antagonistic/additive effects on SW48 (Table 4). The type of interaction between the mixed drugs depends upon their relative concentrations, exposure time and the tested cell line.

The effect of this combination on human colorectal cancer cell cycle was also evaluated. Treatment with Rosco alone growth inhibited colorectal cancer cells in G₂/M phase (24.9% *vs* 4.75% for untreated), while treatment with 5-FU alone growth arrested cancer cells in S-phase (51.9% *vs* 31.5% for untreated). However the combination of 5-FU and Rosco added in sequential, inverted sequential and simultaneous manners growth arrested human colorectal cancer cells in S-phase: 54.8%, 56.8% and 50.7%, respectively, compared to 51.5% in

the S-phase for untreated (Figure 3B). The combination of 5-FU (4.8 × 10⁻⁵ mol/L) and Rosco (15 µg/mL) had synergistic or additive apoptotic effects on SW1116 and SW837 compared to single treatment with 5-FU or Rosco (Figure 3C).

Modulation of doxorubicin cytotoxicity on human colorectal cancer cells by combination with CDKI Rosco

Human colorectal cancer cells were treated with doxorubicin (10⁻¹¹-10⁻⁶ mol/L) or combinations of doxorubicin (10⁻¹¹-10⁻⁶ mol/L) and Rosco (5 or 10 µg/mL) for 96 h. The combination of doxorubicin and Rosco had a very potent anti-proliferative effect on the colorectal cancer cell line SW48 (IC₅₀ = 5.8 × 10⁻¹¹ mol/L, $P = 0.009$) compared to the effect of doxorubicin alone (IC₅₀ = 5.4 × 10⁻⁸ mol/L) (Figure 4A, Table 5). The combination of doxorubicin and 5 µg/mL Rosco slightly increased the growth inhibition exerted on SW1116 and SW837 with IC₅₀ values equal to 2 × 10⁻⁷ mol/L and 4.1 × 10⁻⁸ mol/L, respectively, compared to IC₅₀ values equal to 6 × 10⁻⁷ mol/L and 4.1 × 10⁻⁷ mol/L obtained when SW1116 and SW837 were treated with doxorubicin alone, respectively, (Figure 3A, Table 5). The combination of doxorubicin and 5 µg/mL Rosco increased the sensitivity of SW1116 (3 fold) and SW837 (10 fold) to doxorubicin (Table 5). The difference in growth inhibition produced by treatment of SW1116 ($P = 0.543$) and SW837 ($P = 0.33$) with doxorubicin plus 5 µg/mL was statistically non-significant. The combination of doxorubicin and Rosco (10 µg/mL) produced very potent anti-proliferative effects on SW48 (IC₅₀ = 5.8 × 10⁻¹¹ mol/L, $P = 0.012$), SW1116 (IC₅₀ = 4.5 × 10⁻⁹ mol/L, $P = 0.068$), and SW837 (IC₅₀ = 8.2 × 10⁻¹⁰ mol/L, $P = 0.049$) compared to treatment with Rosco alone (Figure 4A, Table 5). The combination of doxorubicin and Rosco exhibited an additive effect on SW48, synergistic and additive effects on SW1116 and synergistic effect on SW837 in a time and

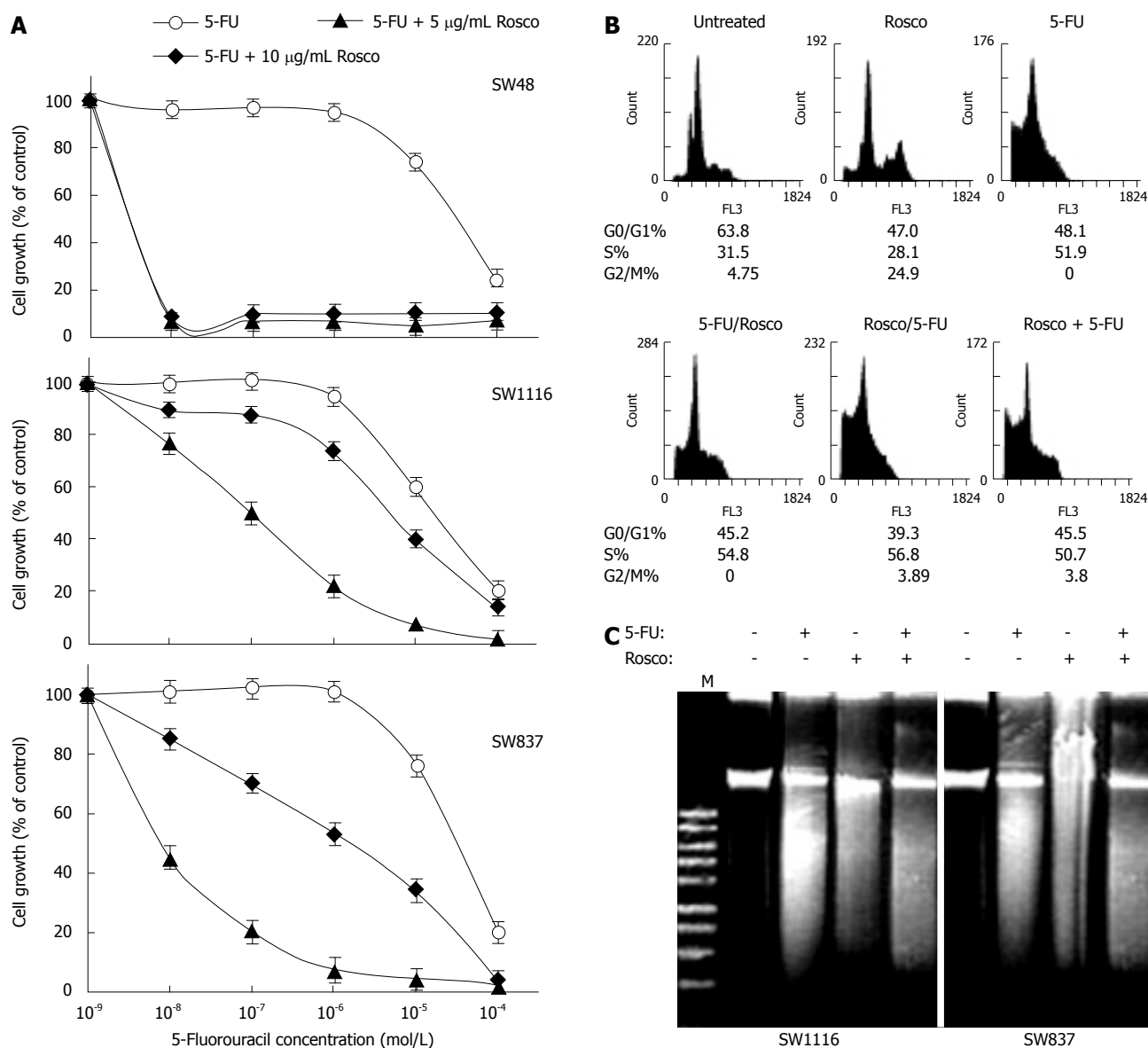


Figure 3 Potentiation of 5-FU anticancer effect on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A:** Human colorectal cancer cell lines were treated with 5-FU (10^{-9} - 10^{-4} mol/L), and the combination of 5-FU (10^{-9} - 10^{-4} mol/L) and Rosco (5 µg/mL or 10 µg/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using the MTT assay; **B:** Cell cycle phase distribution of human colorectal cancer cells (SW837, 5×10^5 cells/well) treated with 5-FU (4.8×10^{-5} mol/L, 72 h); Rosco (15 µg/mL, 72 h); sequential combination: 5-FU (4.8×10^{-5} mol/L, 24 h) followed by Rosco (15 µg/mL, 48 h); inverted sequential combination: Rosco (15 µg/mL, 24 h) followed by 5-FU (4.8×10^{-5} mol/L, 48 h) and simultaneous combination: 5-FU plus Rosco (4.8×10^{-5} mol/L, 15 µg/mL, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated as described above; **C:** Human colorectal cancer cells, SW1116 and SW837 (5×10^5 cells/well), were treated with 5-FU (4.8×10^{-5} mol/L), Rosco (15 µg/mL), and the combination of 5-FU plus Rosco (4.8×10^{-5} mol/L + 15 µg/mL) for 72 h. DNA fragments were extracted and analyzed on 1% agarose gel.

Table 5 IC₅₀ and sensitization ratio of doxorubicin and combinations with Rosco towards human colorectal cancer cell lines

Treatment with doxorubicin and its combinations with Rosco	IC ₅₀ (mol/L)			Sensitization ratio ¹		
	SW48	SW1116	SW837	SW48	SW1116	SW837
Dox.	5.4×10^{-8}	6×10^{-7}	4.1×10^{-7}	-	-	-
Dox. + 5 µg/mL Rosco	5.8×10^{-11}	2×10^{-7}	4.1×10^{-8}	9.3×10^2	3	10
Dox. + 10 µg/mL Rosco	5.8×10^{-11}	4.5×10^{-9}	8.2×10^{-10}	9.3×10^2	1.3×10^2	5.0×10^2

¹Sensitization ratio = IC₅₀ (Dox.)/IC₅₀ (Dox. + Rosco).

dose dependent manner (Table 6).

The effect of the combination on colorectal cancer

cell cycle was also investigated. Treatment with Rosco growth arrested cancer cells in G₂/M (24.9% *vs* 4.75%

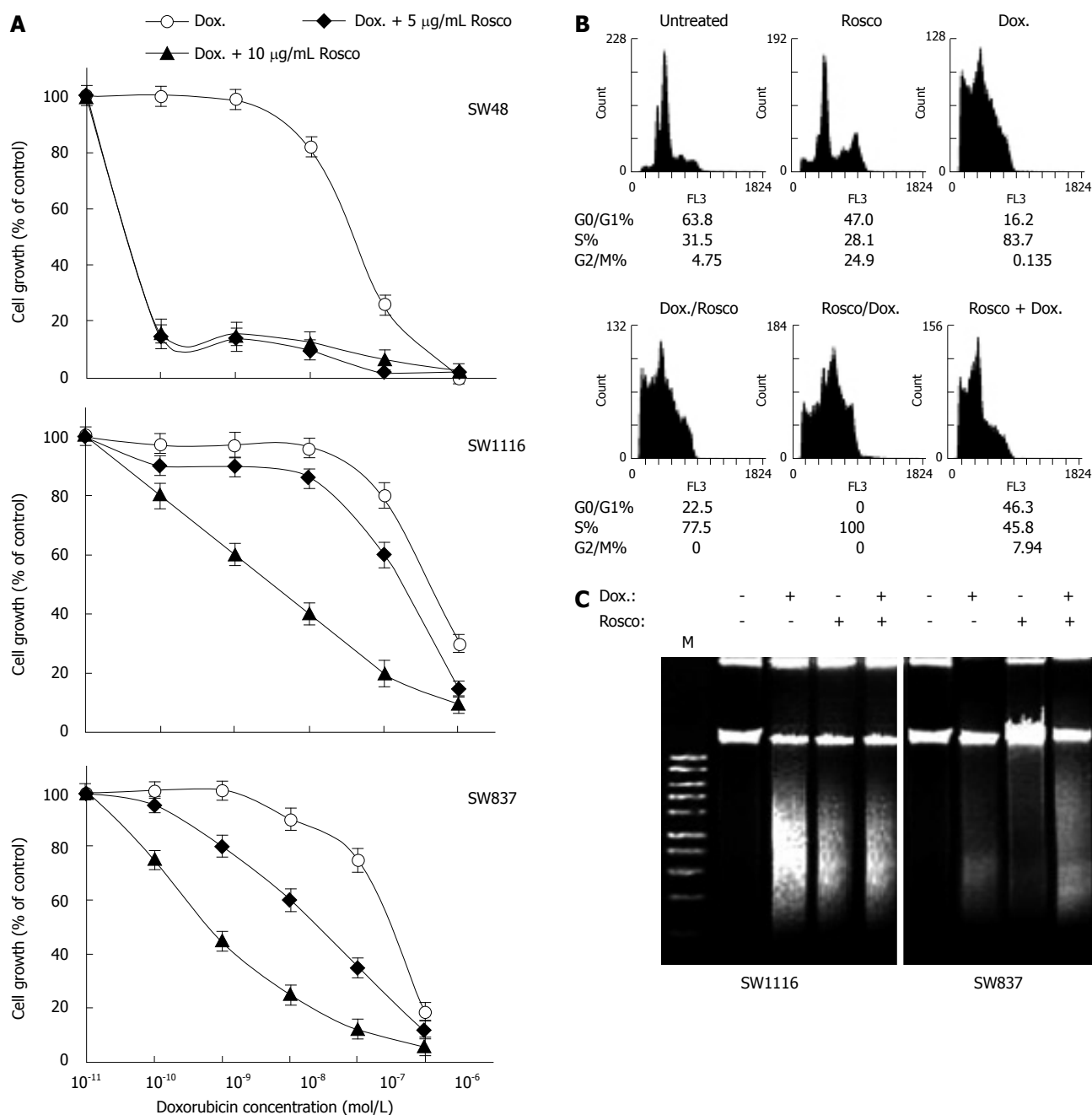


Figure 4 Potentiation of doxorubicin anticancer effect on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A:** Human colorectal cancer cell lines were treated with doxorubicin (10^{-11} - 10^{-6} mol/L), and the combination of doxorubicin (10^{-11} - 10^{-6} mol/L) plus Rosco (5 μ g/mL or 10 μ g/mL) for 96 h. At the end of treatment, control and drug treated cells were scored for proliferation using an MTT assay; **B:** Cell cycle phase distribution of human colorectal cancer cells (SW837, 5×10^5 cells/well) treated with doxorubicin (8×10^{-7} mol/L, 72 h); Rosco (15 μ g/mL, 72 h); sequential combination: doxorubicin (8×10^{-7} mol/L, 24 h) followed by Rosco (15 μ g/mL, 48 h); inverted sequential combination: Rosco (15 μ g/mL, 24 h) followed by doxorubicin (8×10^{-7} mol/L, 48 h) and simultaneous combination: doxorubicin plus Rosco (8×10^{-7} mol/L + 15 μ g/mL, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated as previously described; **C:** Human colorectal cancer cells, SW1116 and SW837, (5×10^5 cells/well) were treated with doxorubicin (8×10^{-7} mol/L), Rosco (15 μ g/mL), and the combination of doxorubicin plus Rosco (8×10^{-7} mol/L + 15 μ g/mL) for 72 h. DNA fragments were extracted and analyzed on 1% agarose gel.

for untreated), while doxorubicin markedly growth arrested colorectal cancer cells in S-phase (83.7% *vs* 51.5% for untreated). The combination of doxorubicin and Rosco added to the culture in a sequential manner, i.e., doxorubicin followed by Rosco, growth arrested cancer cells in S-phase (77.5% *vs* 31.5% for untreated). The same combination added in an inverted sequential manner growth arrested cancer cells completely in S-phase (100% *vs* 31.5% for untreated). However, when the same

combination was added in a simultaneous manner, the colorectal cancer cells were arrested in both S-phase (45.8% *vs* 31.5% for untreated) and G₂/M phase (7.94% *vs* 4.75% for untreated) (Figure 4B). The apoptosis inducing effect of the combination of doxorubicin (8×10^{-7} mol/L) and Rosco (15 μ g/mL) was also tested. This combination enhanced apoptosis in SW1116 and SW837 compared to single treatment of doxorubicin and Rosco (Figure 4C).

Table 6 Analysis of the combined effects of doxorubicin and Rosco on human colorectal cancer cell lines

Combined treatment of doxorubicin and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells ¹								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
Dox 0.5 IC ₅₀ + Rosco 1.0 µg/mL	ant	ant	ant	syn	syn	syn	syn	syn	syn
Dox 1.0 IC ₅₀ + Rosco 10 µg/mL	ant	ant	ant	syn	syn	syn	syn	syn	syn
Dox 2.0 IC ₅₀ + Rosco 15 µg/mL	ant	ant	ant	syn	syn	syn	syn	syn	syn
Dox 3.0 IC ₅₀ + Rosco 20 µg/mL	ant	add	add	syn	syn	add	syn	syn	add
Dox 4.0 IC ₅₀ + Rosco 25 µg/mL	ant	add	add	syn	ant	add	syn	ant	ant

¹The data are based on the mean of absorbance measurements of 3 independent experiments; ant: Antagonistic; add: Additive; syn: Synergistic.

Table 7 IC₅₀ and sensitization ratio of vinblastine and its combinations with Rosco towards human colorectal cancer cell lines

Treatment with vinblastine and combinations with Rosco	IC ₅₀ (mol/L)			Sensitization ratio ¹		
	SW48	SW1116	SW837	SW48	SW1116	SW837
Vinb.	1 × 10 ⁻⁸	6.3 × 10 ⁻⁷	4.2 × 10 ⁻⁷	-	-	-
Vinb. + 5 µg/mL Rosco	5.4 × 10 ⁻¹¹	7.5 × 10 ⁻⁸	5.0 × 10 ⁻⁸	1.9 × 10 ²	8.4	8.4
Vinb. + 10 µg/mL Rosco	5.4 × 10 ⁻¹¹	7.9 × 10 ⁻¹⁰	4.6 × 10 ⁻¹⁰	1.9 × 10 ²	8.0 × 10 ²	9.1 × 10 ²

¹Sensitization ratio = IC₅₀ (Vinb.)/IC₅₀ (Vinb. + Rosco).

Modulation of vinblastine cytotoxicity on human colorectal cancer cells by combination with CDKI Rosco

The ability of cyclin dependent kinase inhibitor Rosco to sensitize human colorectal cancer cells to vinblastine was evaluated. Treatment of cancer cells with a combination of vinblastine (10⁻¹²-10⁻⁷ mol/L) and Rosco (5 or 10 µg/mL) dramatically growth inhibited SW48 cells ($P = 0.023$ or $P = 0.019$) with IC₅₀ = 5.4 × 10⁻¹¹ mol/L compared to IC₅₀ = 1.0 × 10⁻⁸ mol/L for the effect of vinblastine alone (Figure 5). These results clearly indicate that Rosco (5 or 10 µg/mL) sensitized SW48 cells to vinblastine anticancer activity by 190 fold (Table 7). Treatment of SW1116 with the combination of vinblastine and Rosco (5 µg/mL) produced a higher growth inhibition of SW1116 (IC₅₀ = 7.5 × 10⁻⁸ mol/L, sensitization ratio = 8.4 fold) (Figure 5A, Table 7) compared to treatment with vinblastine alone (IC₅₀ = 6.3 × 10⁻⁷ mol/L). The increase in the growth inhibition induced by mixing vinblastine with 5 µg/mL of Rosco was found to be statistically non-significant ($P = 0.216$). On the other hand, treatment of SW1116 with vinblastine and 10 µg/mL Rosco markedly enhanced the cytotoxicity of vinblastine (IC₅₀ = 7.9 × 10⁻¹⁰ mol/L, $P = 0.013$) towards SW1116 demonstrating a great increase in the chemo-sensitization of SW1116 (8 × 10²-fold) to vinblastine (Table 7). Similar results were obtained with the colorectal cancer cell line SW837. The combination of vinblastine and Rosco (5 µg/mL) slightly increased the growth inhibition of SW837 (IC₅₀ = 5 × 10⁻⁸ mol/L, sensitization ratio = 8.4) compared to single treatment with vinblastine (IC₅₀ = 4.2 × 10⁻⁷ mol/L) (Table 7). However, the combination of vinblastine and 10 µg/mL Rosco exerted significant growth inhibition of SW837 cells (IC₅₀ = 4.6 × 10⁻¹⁰ mol/L, $P = 0.023$) compared to treatment with vinblastine alone (IC₅₀ = 4.2 × 10⁻⁷ mol/L) demonstrating a marked increase in the sensitivity of SW837 (9.1 ×

10²-fold) to vinblastine.

The combination of vinblastine (2IC₅₀) and Rosco (15 µg/mL) had synergistic effect on SW48 after 4 d of combination treatment (Table 8). All the tested combinations had additive or synergistic effects on SW1116 after 2 d of treatment. The combination of vinblastine (IC₅₀) and Rosco (10 µg/mL) had additive and synergistic effects on SW1116 and SW837 after 4 and 6 d of treatment, respectively (Table 8).

The effects of single and combined treatment with vinblastine (2.6 × 10⁻⁷ mol/L) and Rosco (15 µg/mL) on colorectal cancer cell cycle distribution indicated that treatment of SW837 with Rosco growth inhibited colorectal cancer cells in G₂/M (24.9% *vs* 4.75% for untreated), while, vinblastine treatment markedly growth arrest these cells in G₂/M (71.9% *vs* 4.75% for untreated). The combination of vinblastine (2.6 × 10⁻⁷ mol/L) and Rosco (15 µg/mL) added in sequential manner (vinblastine followed by Rosco) greatly growth arrested SW837 cells in G₂/M (76.6% *vs* 4.75% for untreated). The same combination added in an inverted sequential manner markedly growth arrested the cells in G₂/M (85.7% *vs* 4.75% for untreated). The same combination added simultaneously to SW837 cells growth arrested these cells both in S- (44.6% *vs* 31.6% for untreated) and G₂/M-(19.3% *vs* 4.75% for untreated) phases (Figure 5B). The combination of vinblastine (2.6 × 10⁻⁷ mol/L) and Rosco (15 µg/mL) had a marked apoptotic effect on colorectal cancer cell lines SW1116 and SW837 compared to single treatments with vinblastine (2.6 × 10⁻⁷ mol/L) or Rosco (15 µg/mL) (Figure 5C).

DISCUSSION

The central finding of the present study is that the

Table 8 Analysis of the combined effects of vinblastine and Rosco on human colorectal cancer cell lines

Combined treatment of vinblastine and Rosco	Combination interaction at various durations of treatment in human colorectal cancer cells ¹								
	SW48			SW1116			SW837		
	2 d	4 d	6 d	2 d	4 d	6 d	2 d	4 d	6 d
Vinb 1.0 IC ₅₀ + Rosco 10 µg/mL	ant	ant	ant	add	add	add	ant	syn	syn
Vinb 2.0 IC ₅₀ + Rosco 15 µg/mL	ant	syn	ant	syn	ant	ant	ant	ant	ant
Vinb 3.0 IC ₅₀ + Rosco 20 µg/mL	ant	ant	ant	syn	ant	ant	ant	ant	ant
Vinb 4.0 IC ₅₀ + Rosco 25 µg/mL	ant	ant	ant	syn	ant	ant	ant	ant	ant

¹The data are based on the mean of absorbance measurements of 3 independent experiments; ant: Antagonistic; add: Additive; syn: Synergistic.

cyclin dependent kinase inhibitor Rosco improved the therapeutic activity of several conventional chemotherapeutic drugs namely taxol, doxorubicin, 5-FU, and vinblastine that act by different mechanisms in human colorectal cancer cells. This finding is significant because chemotherapeutic drugs cause high toxicity to normal tissues during treatment of colorectal cancer as well as other cancers.

The adverse health effects of the conventional chemotherapeutic drugs, such as immunosuppression and cardiomyopathy, which severely increase in a dose-dependent manner, as well as development of primary or secondary drug resistance in tumor cells, limit their clinical success in cancer chemotherapy^[15]. The increase in systemic toxicity and drug resistance, the major drawbacks of anticancer chemotherapeutic agents, has led to a new challenge in the field of cancer research. To overcome such problems, extensive research has been directed towards reducing systemic toxicity and increasing drug activity in cancer therapy^[15,16]. In this regard, combination chemotherapy has received increasing attention in the search for compounds that could increase the therapeutic index of clinical anticancer drugs^[17].

Deregulation of the cell cycle and oncogenic overexpression of several cell cycle related gene products in many human cancers provide new opportunities for anticancer drug discovery. Efforts to exploit these targets are progressing quite well, with inhibition of cyclin dependent kinase activity emerging as the most productive approach at present. This has resulted in the development of several small molecules, with specific and potent CDK inhibitory effects, which are now undergoing clinical trials in phases I and II, and the results awaited with expectation.

CDKs are essential players in the intracellular control of the cell cycle. Since CDKs and their regulatory partners are frequently deregulated and exhibit enhanced activity in human cancers, their inhibition by selectively acting drugs offers a new concept in the therapeutic strategy^[5,18]. Recently, a number of pharmacological inhibitors of CDKs were developed, one efficient group of such compounds is based on the substitution of purines and pyrimidines. Substituted purines represent CDK inhibitors that are structurally most similar to ATP^[7]. Among a series of C2, N6, N9-substituted adenines, Rosco displays high efficiency and selectivity towards some CDKs. Out of 25 kinases investigated,

only a few were significantly inhibited by Rosco with IC₅₀ values lower than 1 µmol/L. CDK2/cyclin B and CDK2/cyclin A were identified as the best targets^[8]. Through its high selectivity, Rosco is predestinated to be a potent anti-mitotic drug. It acts not only as a cell cycle blocker^[19-22], but seems also to induce apoptosis^[11,22-24].

There is still a lack of systematic knowledge about the cytotoxic effects of Rosco on normal and malignant cells. The exact discrimination between inhibition of cell proliferation and impairment of cell viability is necessary. Moreover, the pro- or anti-apoptotic action of Rosco on different cells is until now contradictory and has to be conscientiously examined. The consequences of Rosco-induced cell cycle arrest and apoptosis in colorectal cancer cells upon chemo-sensitization of such cells to conventional therapeutic drugs have not yet been investigated. In this study, we addressed the ability of Rosco to synergize with conventional chemotherapeutic drugs, acting by different mechanisms, to induce efficient apoptosis in human colorectal cancer cell lines.

In the present study, human colorectal cancer cells were shown to be sensitive to the antiproliferative and cytotoxic effects of Rosco with IC₅₀ values: (11.56-25 µg/mL), (11.62-14.78 µg/mL) and (13.44-16.25 µg/mL) for SW48, SW1116 and SW837, respectively, after 24-144 h, 72-144 h and 48-144 h of Rosco treatment, respectively. These results are consistent with those reported for other cell types^[8,25]. To explore whether inhibition of cell growth observed in the Rosco treated human colorectal cancer cells synergize with the conventional chemotherapeutic drugs acting by different mechanisms to induce efficient apoptosis of human colorectal cancer cells, we tested the efficacy of single and combined treatments with Rosco and taxol, doxorubicin, 5-FU or vinblastine on the growth of human colorectal cancer cells. Our results indicated that Rosco (5 and 10 µg/mL) markedly sensitized the tested human colorectal cancer cells to taxol (sensitization ratio = $7.0-6.4 \times 10^3$), doxorubicin (sensitization ratio = $3.0-9.3 \times 10^3$), 5-FU (sensitization ratio = $3.73-8.42 \times 10^3$) and vinblastine (sensitization ratio = $9-8 \times 10^3$) (Tables 1, 3, 5 and 7). The combination of Rosco and chemotherapeutic drugs inhibited the growth of human colorectal cancer cells in an additive or synergistic fashion, and in a time- and dose-dependent manner (Tables 2, 4, 6 and 8). Treatment of human colorectal cancer cells with Rosco, conventional chemotherapeutic drugs or the combination of Rosco and conventional chemotherapeutic drugs add-

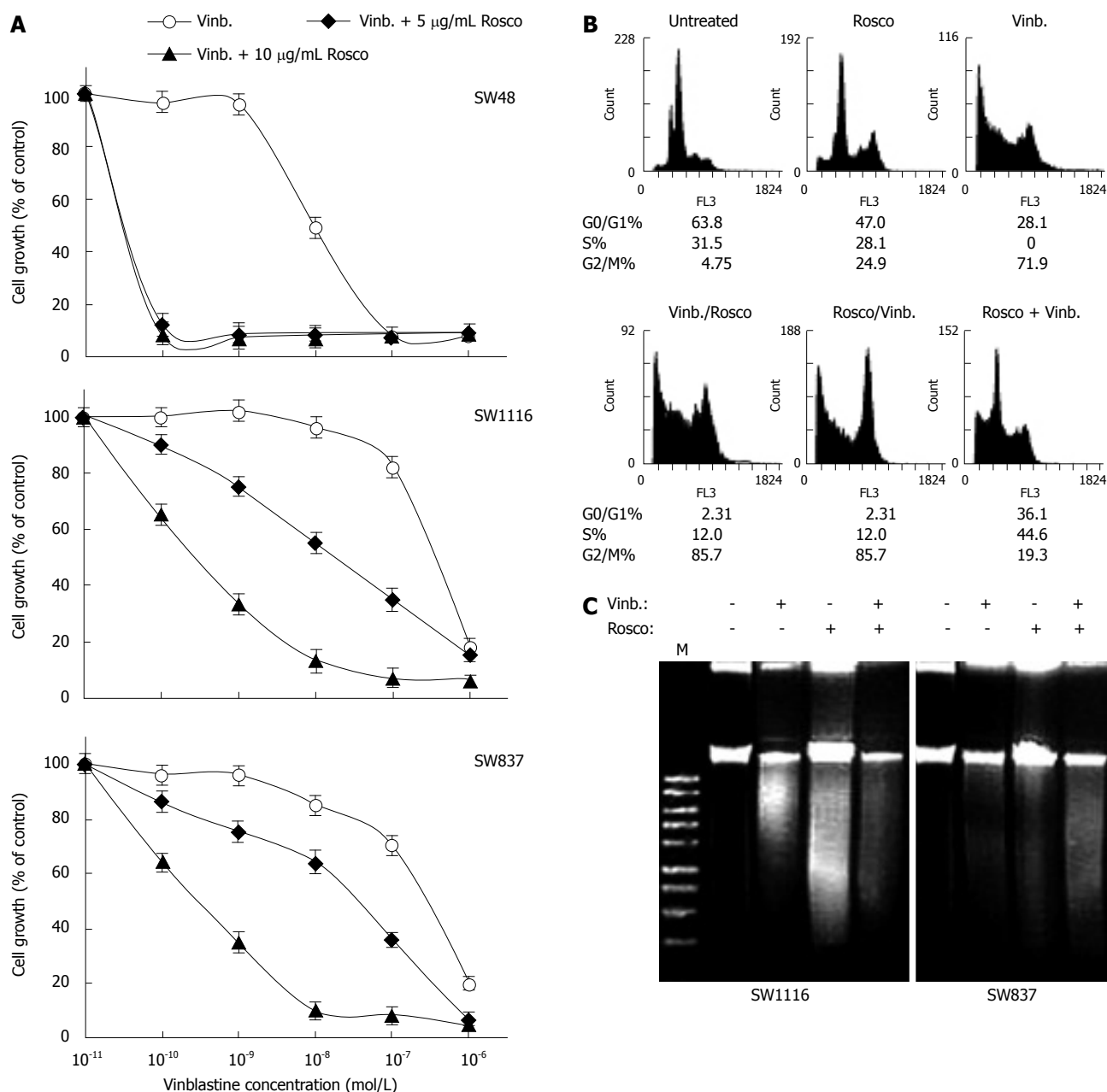


Figure 5 Potentiation of vinblastine anticancer effect on human colorectal cancer cell lines by combination with cyclin dependent kinase inhibitor roscovitine. **A:** Human colorectal cancer cell lines were treated with vinblastine (10^{-12} - 10^{-7} mol/L) and the combination of vinblastine (10^{-12} - 10^{-7} mol/L) plus Rosco (5 $\mu\text{g/mL}$ or 10 $\mu\text{g/mL}$) for 96 h. At the end of treatment, control and drug-treated cells were scored for proliferation using an MTT assay; **B:** Cell cycle phase distribution of human colorectal cancer cells (SW837, 5×10^5 cells/well) treated with vinblastine (2.6×10^{-7} mol/L, 72 h); Rosco (15 $\mu\text{g/mL}$, 72 h); sequential combination: vinblastine (2.6×10^{-7} mol/L, 24 h) followed by Rosco (15 $\mu\text{g/mL}$, 48 h); inverted sequential combination: Rosco (15 $\mu\text{g/mL}$, 24 h) followed by vinblastine (2.6×10^{-7} mol/L, 48 h) and simultaneous combination: vinblastine plus Rosco (2.6×10^{-7} mol/L + 15 $\mu\text{g/mL}$, 72 h) was evaluated by flow cytometry. The percentage of cells in different phases of the cycle was calculated as described above; **C:** Human colorectal cancer cells, SW1116 and SW837, (5×10^5 cells/well) were treated with vinblastine (2.6×10^{-7} mol/L), Rosco (15 $\mu\text{g/mL}$) and the combination of vinblastine plus Rosco (2.6×10^{-7} mol/L + 15 $\mu\text{g/mL}$) for 72 h. DNA fragments were extracted and analyzed on 1% agarose gel.

ed in a sequential or inverted sequential manner growth arrested colorectal cancer cells in G_2/M - or S- phase of the cell cycle. While, simultaneous addition of Rosco and conventional chemotherapeutic drugs double blocked colorectal cancer cells in G_2/M - and S- phases of the cell cycle for all the tested drugs except for 5-FU, where, its combination with Rosco growth arrested colorectal cancer cells in S-phase. The growth arrest of colorectal cancer cells in G_2/M following Rosco treatment may facilitate the induction of apoptosis and sensitize the cells to conven-

tional chemotherapeutic drugs.

Another notable observation from our morphological analysis was the extensive detachment of cells from the cell culture substratum after exposure to Rosco (data not shown). Recent evidence suggests that cellular attachment to the substratum is mediated by the interaction of integrins with ECM components such as fibronectin, collagen, and vitronectin^[26]. Binding of integrins to these adhesion molecules results in the activation of focal adhesion kinase^[27] accompanied by phosphorylation and recruit-

ment of a number of related cytoskeletal and signaling molecules, thereby transducing anchorage and survival messages to the nucleus^[28,29]. Conversely, the uncoupling of integrins from ECM proteins leads to disruption of integrin-mediated signal transduction, inactivation of focal adhesion kinase, detachment of cells from the ECM, and apoptotic cell death^[30]. Our data suggest that following Rosco treatment, human colorectal cancer cells detach from cell culture substratum, and die *via* apoptosis as indicated by the DNA fragmentation assay. This notion is consistent with previous reports demonstrating that cells deprived of matrix attachment underwent apoptosis^[31]. Thus, the extensive detachment of cells from the cell culture substratum, and the apoptotic cell death observed in our experimental system may be due to the uncoupling of integrin-mediated signaling and/or disruption of cell-matrix interactions induced by Rosco. In addition to facilitating apoptosis, which will have its impact on chemosensitization of human colorectal cancer cells to conventional chemotherapeutic drugs, the loss of adhesion induced by this CDK inhibitor may deny cell anchorage and traction necessary for growth and migration and thus prevent colorectal cancer invasion and metastasis, the major cause of death in colorectal cancer patients. Because adhesion and invasion are crucial to the initiation of metastatic growth^[32], additional studies on the effect of Rosco on cell adhesion to extracellular matrix components as well as the anti-invasive potential of the CDK inhibitor could be extremely rewarding. These studies are currently ongoing in our laboratory.

Rosco may prevent the assembly of actin fibers by modulating the expression and/or activity of Rho GTPases, which have been reported to be involved in the regulation of actin microfilament organization and other associated activities^[33]. Disruption of actin microfilament architecture by Rosco has some biological implications. In view of the role played by actin microfilaments in various aspects of cellular physiology such as cell-cell interaction, proliferation, and secretion^[33], it can be argued that all of these cellular activities could be affected in colorectal tumors following Rosco treatment.

Rosco strongly up-regulates wt p53 protein in cancer cells^[19,20,22,34]. Since p53 protein plays a pivotal role in the regulation of cell cycle, the biological effect of Rosco cannot be restricted to the direct inhibition of distinct kinases. Considering the multiple p53 targets and functions, it is obvious that the Rosco-induced upregulation of p53 in cancer cells may essentially contribute to the cell cycle arrest, chromatin silencing and initiation as well as execution of apoptosis.

The role of CKDs in chemosensitization, and the potential downstream effectors of CDKs inhibition have been investigated by Crescenzi *et al.*^[5]. They showed that lung adenocarcinoma cell line H1299 treated with a nontoxic concentration of Rosco renders H1299 cells significantly more susceptible to doxorubicin or etoposide. In these cells, Rosco does not modulate senescence, but markedly reduces the capacity of H1299 cells to repair damage and resume proliferation after treatment.

Combined treatment with Rosco and doxorubicin, or etoposide was found to enhance G₂-M accumulation, to increase the amount of γ -H2AX foci and to inhibit DNA repair. Two main repair pathways, homologous recombination and NHEJ, cooperate to repair DNA DSBs^[35]. Crescenzi *et al.*^[5] investigated the ability of Rosco to modulate those two processes in doxorubicin-treated cells. They reported the ability of Rosco to negatively modulate DNA-PK activity in H1299 cells^[5,36] and showed that Rosco significantly reduces the efficiency of recombination repair identifying a novel mechanism of action by which Rosco affects tumor cells that is inhibition of DNA DSBs repair.

The role of CDK2 and CDK1 kinases as targets for Rosco in tumor chemosensitization has also been investigated by Crescenzi *et al.*^[5]. In this study, experiments with inducible dn-K2 clones indicated that loss of Cdk2 and Cdk1 activity was responsible for the chemosensitizing effect of Rosco. Overexpression of dn-K2 in H1299 cells potentiates doxorubicin-induced G₂-M arrest and inhibited recovery of the cells after treatment. It is worth noting that overexpression of dn-K2 results in both Cdk2 and Cdk1 inhibition^[37]. Furthermore, analyses of homologous recombination in HeLa cells transiently overexpressing either dn-K2 or dn-K1 or Cdk2 confirmed a role for Cdk2 in modulation of DNA repair processes^[5]. A role for CDK in the control of DNA repair pathways has also been studied in the yeast cells^[38,39]. Combined treatment of Rosco and DNA-damaging agents not only enhances drug-induced apoptosis, but also effectively hampers the recovery of mildly damaged tumor cells after treatment. Rosco, by hindering both homologous recombination and NHEJ repair processes, has the potential to inhibit recovery of mildly damaged tumor cells after chemotherapeutic drug treatment, and to increase the susceptibility of tumor cells to chemotherapy. Our results clearly indicated that Rosco synergizes with chemotherapeutic drugs to induce efficient apoptosis of human colorectal cancer cells. Important issues that need to be addressed in order to advance these agents to the clinical arena include the best drug administration schedule, testing various combinations with standard chemotherapeutic agents, the best tumor types to be targeted, and demonstration of CDK modulation of tumor samples from cancer patients.

COMMENTS

Background

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that play a key role in regulating cell cycle progression. Aberrant expression or altered activity of distinct CDK complexes results in escape of cells from the cell cycle control and leads to malignant transformation. Therefore, inhibition of CDKs in malignant cells provides a new strategy in the fight against cancer. The present study examined the ability of roscovitine (Rosco), a CDK inhibitor (CDKI), to enhance the anticancer effects of chemotherapeutic drugs that act by different mechanisms on human colorectal cancer cells. The authors have also investigated whether Rosco differentially affects the cell cycle distribution of drug-treated human colorectal cancer cells.

Research frontiers

Extensive research has been directed towards reducing systemic toxicity and

increasing drug activity in cancer therapy. Combination chemotherapy has received increasing attention in the search for compounds that could increase the therapeutic index of clinical anticancer drugs. This study indicated that combinations of agents directed at different pathways or different steps of pathways involved in apoptosis can cause the cells to reach an apoptosis threshold resulting in synergistic apoptosis and increased therapeutic index of the anticancer drugs.

Innovations and breakthroughs

The central finding of this study is that the cyclin dependent kinase inhibitor Rosco improved the therapeutic activity of several conventional drugs namely taxol, 5-fluorouracil (5-FU), doxorubicin and vinblastine that act by different mechanisms. Also, Rosco differentially affected the cell cycle distribution of drug-treated colorectal cancer cells.

Applications

Chemotherapeutic drugs are highly toxic to normal tissues during treatment of colorectal cancer as well as other cancers. Rosco increases the sensitivity of several conventional chemotherapeutic drugs namely taxol, doxorubicin, 5-FU, and vinblastine. This finding is significant because increasing drug activity may reduce systemic toxicity in cancer therapy.

Terminology

CDKs are a heterogeneous group of compounds that are able to inhibit CDKs involved in the cell cycle, transcription or neuronal functions. CDKs are a chemically diverse, flat, hydrophobic heterocycles that compete with ATP. Rosco (CDKI) is structurally related to ATP, it blocks the cell cycle and induces apoptosis.

Peer review

A tremendous amount of *in vitro* work has been done looking at the anti colorectal cancer cell effect of Rosco, a novel CDK inhibitor, to enhance the antitumor effects of conventional chemotherapy agents. The approach is very well designed and the presentation of data is very detailed. It looks promising and may yield clinical benefits in the future.

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BASIC RESEARCH

Enhanced therapeutic effects for human pancreatic cancer by application K-ras and IGF-IR antisense oligodeoxynucleotides

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Abstract

AIM: To investigate the combined effects of K-ras antisense oligodeoxynucleotide (K-ras ASODN) specific to GTT point mutation at codon 12 and type I insulin-like growth factor receptor (IGF-IR) antisense oligodeoxynucleotide (IGF-IR ASODN) on proliferation and apoptosis of human pancreatic cancer Patu8988 cells *in vitro* and *in vivo*.

METHODS: K-ras gene point mutation and its style at codon 12 of human pancreatic cancer cell line Patu8988 were detected by using polymerase chain reaction with special sequence primers (PCR-SSP) and sequence analysis. According to the mutation style, K-ras mutation ASODN specific to K-ras point mutation at codon 12 was designed and composed. After K-ras ASODN and IGF-IR ASODN treated on Patu8988 cells respectively or cooperatively, the proliferation and morphological change of Patu8988 cells were analyzed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, colony

forming assay and transmission electron microscopy; the expression of K-ras and IGF-IR mRNA and protein in the treated cells was measured by reverse-transcript polymerase chain reaction (RT-PCR) and flow cytometry respectively; apoptosis was determined by flow cytometry. The combined antitumor activity of K-ras ASODN and IGF-IR ASODN was evaluated in BALB/c nude mice bearing human pancreatic cancer inoculated with Patu8988 cells.

RESULTS: The results of PCR-SSP and sequence analysis showed that the human pancreatic cancer cell line Patu8988 had point mutation at codon 12, and the mutation style was GGT→GTT. 2-32 µg/mL K-ras ASODN and 2-32 µg/mL IGF-IR ASODN could inhibit Patu8988 cells' growth, induce apoptosis and decrease the expression of K-ras and IGF-IR mRNA and protein alone. However, there was much more effective inhibition of growth and induction of apoptosis by their combination than by each one alone. In tumor bearing mice, the combination of K-ras ASODN and IGF-IR ASODN showed a significant inhibitory effect on the growth of transplanted pancreatic cancer, resulting in a statistically significant difference compared with each alone.

CONCLUSION: It has been found that K-ras ASODN combined with IGF-IR ASODN could cooperatively inhibit the growth of Patu8988 cells, and induce their apoptosis *via* reinforcing specific down regulation of K-ras and IGF-IR mRNA and protein expression.

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Key words: Pancreatic cancer; Antisense oligodeoxynucleotide; K-ras; Type I insulin-like growth factor receptor; Patu8988

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INTRODUCTION

Pancreatic cancer is the fatal cancer of the digestive system with the worst prognosis. The 5-year survival rate is approximately 1%-2%, and the median survival time after diagnosis ranges only 4-6 mo^[1-3]. The reasons for poor prognosis include: (1) the difficulty of early diagnosis due to its anatomical location, and lack of specific early syndromes; (2) the high potential to infiltrate to the surrounding tissues and metastasize even in the early stage; and (3) the poor responsiveness to conventional treatments such as chemotherapy, radiotherapy and immunotherapy^[4-6]. Surgery represents the only opportunity for possible cure, but it is restricted to early stage pancreatic cancer and most patients who undergo tumor resection show recurrence or distant metastases and die within a few years. At least, at the present time, an emphasis on early diagnosis alone may not be sufficient for significant improvement in the current poor prognosis of pancreatic cancer, which necessitates the search for novel treatment strategies to improve the prognosis.

Previous studies have demonstrated that a high percentage of pancreatic cancers harbors *K-ras* gene point mutation and overexpresses insulin-like growth factor receptor type 1 (IGF-IR)^[7-11]. These alterations may together contribute to the progression and aggressiveness of pancreatic cancer from different pathways. Consequently, targeting expression of *K-ras* or IGF-IR has a potential value in pancreatic cancer therapy, and has led to the development of new therapeutic strategies based on the use of agents able to selectively inhibit targeted gene expression. In particular, antisense oligodeoxynucleotides (ASODNs) have proved their efficacy as targeted therapy, and are able to modulate target protein expression in pancreatic cancer studies^[12-15]. In the practical application of the ASODN approach, many key problems need to be solved: selection of a single agent does not seem particularly promising because of the multigenic alterations of pancreatic cancer; finding a targeting site of *K-ras* mRNA or *IGF-IR* mRNA that is likely to be accessible to ASODNs; selection of an adaptable vector for mediating ASODNs; optimization of transfection concentration in a cell line, *etc.* Based on these considerations, we used polymerase chain reaction with special sequence primers (PCR-SSP) and sequence analysis to detect a *K-ras* point mutation at codon 12, and its mutation style on pancreatic cancer Patu8988 cells, designed and prepared ASODN (*K-ras* ASODN) specific for the *K-ras* point mutation at codon 12, and then combined it with strongest efficient IGF-IR ASODN designed by Resnicoff *et al.*^[16] to transfect pancreatic cancer Patu8988 cells with highly efficient vector Lipofectamine 2000. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse-transcript polymerase chain reaction (RT-PCR), flow cytometry and transmission electron microscope were used to evaluate the effects of cell proliferation, apoptosis and target gene expression. Therapeutic

efficacy of the combination treatment was also evaluated in xenografts.

MATERIALS AND METHODS

Cell culture

Human pancreatic cancer cell lines Patu8988 and BXPC-3 used in this study were preserved in our laboratory. The cells were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Hangzhou, China), 5 mmol/L HEPES, 100 U/mL penicillin and 100 U/mL streptomycin in 5% atmospheric CO₂ at 37°C. Cells were passaged every 3 d, checked routinely, and found to be free of contamination. When cells grew to 75% confluence, they were digested and used for *in vitro* and *in vivo* studies.

K-ras gene point mutation at codon 12 detected by PCR-SSP and sequence analysis

Genomic DNAs for Patu8988 cells and BXPC-3 cells were extracted according to the protocol. With regard to the sequences of *K-ras* cDNA in Genbank and the three high frequency mutation styles (CGT, GTT and GAT) at codon 12, three kinds of special sequence primers (SSP) for polymerase chain reaction were designed to detect *K-ras* gene point mutation at codon 12 for Patu8988 cells and BXPC-3 cells. Primers were as following: R1: 5'-GGTAGTTGG-AGCTC-3'; R2: 5'-GTAGTTGGAGCTGT-3'; R3: 5'-GTAGTTGGAGCTGA-3'; R4: 5'-CTATTGTTGGA TCATATTCG-3'. The pairing of R1-R4 amplified CGT mutation with a 89 base pair fragment; the pairing R2-R4 amplified GTT mutation with a 88 base pair fragment; the pairing R3-R4 amplified GAT mutation with a 88 base pair fragment. The amplification products were loaded on 8% acrylamide gels, and stained with ethidium bromide to detect mutation styles. In addition, *K-ras* gene was amplified from Patu8988 cells and BXPC-3 cells using RT-PCR, and the PCR products were directly sequenced.

Proliferation assay of Patu8988 cells treated with *K-ras* ASODN or IGF-IR ASODN alone

Based on the results of PCR-SSP and sequence analysis, the antisense phosphorothioate oligodeoxynucleotides 5'-TACGCCAACAGCTCCAAC-3' (*K-ras* ASODN) specific to the *K-ras* gene point mutation at codon 12 were designed and synthesized. The antisense phosphorothioate oligodeoxynucleotides 5'-TCCTCCGGAGCCAGACTT-3' (IGF-IR ASODN) specific to *IGF-IR* gene were synthesized according to the report from Resnicoff *et al.*^[16]. Exponentially growing Patu8988 cells at 1×10^5 /well were seeded in 96-well microtiter plate, and treated with *K-ras* ASODN or IGF-IR ASODN mediated by LipofectamineTM 2000 at concentration of 2-32 mg/L for 24, 48, 72 and 96 h. The culture medium was changed every 24 h with fresh RPMI 1640 medium, which contained the same

concentration of K-ras ASODN or IGF-IR ASODN. The control cultures were left untreated at 37°C for the same period of time, with triplicate wells for each concentration. After incubating for 24, 48, 72 and 96 h, 20 μ L of 5 g/L MTT (Sigma, USA) in PBS was added to each well, followed by incubation for 4 h at 37°C. Formazan crystals were dissolved in DMSO for 15 min at 37°C. Absorbance was determined with an enzyme-linked immunosorbent assay reader at 570 nm. The cell proliferation curves were drawn according to the absorbance. The optimal concentration able to inhibit cell growth was selected for further experiments.

Proliferation assay of Patu8988 cells treated with combination of K-ras ASODN and IGF-IR ASODN

Patu8988 cells were seeded in a 96-well plate at a concentration of 1×10^5 /well, and divided into three groups: (1) 16 mg/L K-ras ASODN group; (2) 16 mg/L IGF-IR ASODN group; (3) 16 mg/L K-ras ASODN + 16 mg/L IGF-IR ASODN group. The cell cultures were measured for cell proliferation at different time points (0, 24, 48, 72 and 96 h after transfection) using MTT assay as described above. The cell proliferation curves were drawn according to the absorbance.

Apoptosis detection by annexin V-FITC/PI dual staining

Cells at the concentration of 1×10^5 /mL were plated in 6-well plates, divided into three groups as described above. After being incubated for 48 h at 37°C, cells were harvested by trypsinization and rinsed with cold PBS twice. After centrifugation, cells were suspended by 250 μ L conjugated buffer solution and then treated with 5 μ L Annexin V-FITC and 10 μ L propidium iodide (PI) for 15 min in the dark at room temperature. Finally, each sample was added into 300 μ L of conjugated buffer solution and analyzed with flow cytometry. The experiments were performed in triplicate and the results were given as mean \pm SE.

K-ras or IGF-IR protein expression detected by flow cytometry

Patu8988 cells, treated as described above, were removed from the plate by brief trypsinization with 0.25% trypsin, and then washed with PBS twice, stained with primary K-ras Ab or IGF-IR Ab, followed by FITC-conjugated goat anti-mouse IgG. After two rinses with PBS containing 2% FBS, these cells were analyzed with flow cytometry. Controls consisted of incubation with no antibodies or incubation with only the secondary antibody. The experiment was repeated three times.

K-ras or IGF-IR mRNA expression detected by semi-quantitative RT-PCR

Cells were plated in 6-well plates and performed as described above. Total cellular RNA was extracted by using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and concentration were determined by measuring the absorbance (A_{260}) at 260 nm and 280 nm (A_{260}/A_{280}). To

generate first-strand cDNA, an oligo (dT) 18 was used as primer, and 2 μ g RNA was reverse-transcribed in the light of MMLV First Strand cDNA Synthesis Kit (Fermentas, USA) protocols. Amplification of human β -actin served as an internal control. The primers used were 5'-GGACCTGACTGACTACCTC-3' (forward) and 5'-TCATACTCCTGCTTGCTG-3' (reverse). The amplification products were 540 bp. The primers for K-ras were 5'-CGCGGATCCATGACTGAATATAACTTGTG-3' (forward) and 5'-CGCAAGCTTTTACATAATTACACACTTTGT-3' (reverse). The amplification products were 585 bp. The primers for IGF-IR were 5'-CCAAAAGTGAAGCCGAGAAG-3' (forward) and 5'-TGCAGCTGTGGATATCGATG-3' (reverse). The amplification products were 300 bp. K-ras was amplified 35 cycles under the following conditions: denaturing at 94°C for 5 min followed by 94°C for 1 min, annealing at 51°C for 30 s and extension at 72°C for 1 min; the final extension was at 72°C for 10 min. IGF-IR gene and β -actin were amplified 30 cycles under the following conditions: denaturing at 94°C for 5 min followed by 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 90 s; the final extension was at 72°C for 10 min. PCR products were separated in 1.5% agarose gels, stained with ethidium bromide, and visualized by UV absorption. Densitometric scanning of the bands was performed, and the relative amount of each gene mRNA expression was estimated by normalization to the β -actin mRNA detected in the same sample.

Transmission electron microscopic examination

Patu8988 cells treated with the combination of K-ras ASODN and IGF-IR ASODN for 48 h were harvested, and washed in PBS. The cell pellets were prefixed in 2.5% glutaraldehyde, postfixed in 1% osmic acid, dehydrated in gradient acetone and embedded in the resin. Ultrathin sections were cut, stained with lead citrate and assessed for the morphological changes under transmission electron microscope.

Colony assays

Twenty Patu8988 cells treated with 16 mg/L K-ras ASODN, 16 mg/L IGF-IR ASODN or 16 mg/L K-ras ASODN + 16 mg/L IGF-IR ASODN were seeded in 6-well plate and cultured in 5% atmospheric CO₂ at 37°C for 2 wk. The control was with the same volume of culture medium.

Treatment in vivo

To investigate whether the combination of K-ras ASODN with IGF-IR ASODN would alter the tumorigenicity of Patu8988, male 4-wk -old BALB/c nude mice were purchased from the Animal Center of Shanghai. 1×10^7 cells in 0.1 mL PBS were injected subcutaneously into the right flank of nude mice. Fourteen days later, 16 mice with about the same tumor size were divided into four groups randomly. Intratumoral injections were given with K-ras ASODN, IGF-IR ASODN or K-ras ASODN + IGF-IR

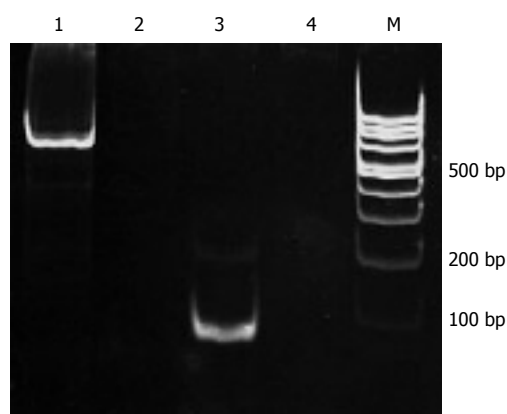


Figure 1 Detection of K-ras point mutation at 12 codon and its style in pancreatic cancer cell line Patu8988 by PCR-SSP. 1: β -actin; 2: R1-R4 pairing (CGT mutation); 3: R2-R4 pairing (GTT mutation); 4: R3-R4 pairing (GAT mutation); M: 100 bp DNA marker.

ASODN, and the control with 100 μ L physiological saline. The injection was repeated every 48 h and 5 times in all. Tumor sizes were measured every 7 d and calculated by the formula: volume (mm^3) = $1/2(\text{width})^2 \times \text{length}$. After a 49-d follow-up period, mice were sacrificed. The tumors were removed, fixed by 4% polyformaldehyde, paraffin embedded and sectioned for immunohistochemical analysis.

Statistical analysis

All experiments were performed in triplicate and data were expressed as mean \pm SD. Statistical analyses were conducted by one-factor analysis of variance and performed with SPSS 10.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

K-ras point mutation at codon 12 of Patu8988 cell line

Detection of K-ras point mutation at codon 12 in the pancreatic cancer cell line Patu8988 is shown as Figure 1. The pairing R2-R4 had the amplification product of GTT mutation with an 88 bp fragment. But, the pairings of R1-R4 and R3-R4 had no amplification product of any mutation. Therefore, K-ras point mutation at codon 12 was found in pancreatic cancer cell line Patu8988, and the mutation style was GTT; no other mutation styles were found. For wild type pancreatic cancer cell line BXCP-3, no amplification products were found in pairings of R1-R4, R2-R4 and R3-R4. The direct sequencing results were consistent with the results from the PCR-SSP (Figure 2).

Inhibition of Patu8988 cell proliferation by K-ras ASODN and IGF-IR ASODN alone or combination

As shown in Figure 3A and B, when Patu8988 cells were exposed to K-ras ASODN and IGF-IR ASODN respectively, the growth of the cells was suppressed as compared to untreated cells ($P < 0.01$) except at the concentration of 2 mg/L. Moreover, when cells were exposed to different doses of K-ras ASODN

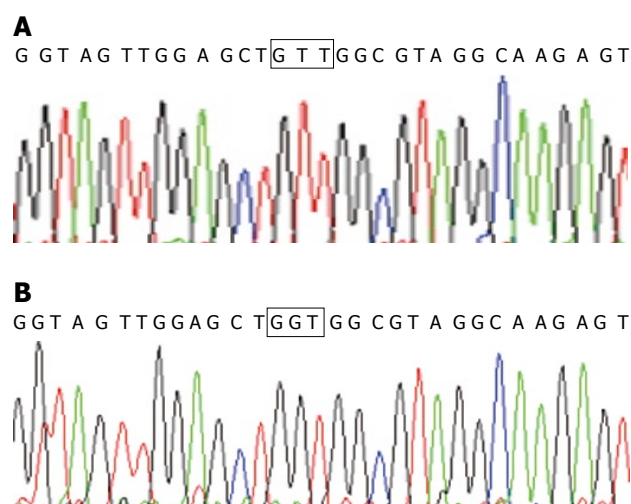


Figure 2 Sequence analysis of pancreatic cancer cell line Patu8988 (A) and BXCP-3 (B). Patu8988 had K-ras point mutation at codon 12 and its style was GGT→GTT (indicated in the square). BXCP-3 was a K-ras gene wild type cell line with normal GGT at codon 12; No other mutation styles were found.

and IGF-IR ASODN individually, growth inhibition was dose dependent: obvious inhibition was seen at the concentration of 16 mg/L, and the greatest effect was seen at a concentration of 32 mg/L. However, no statistical significance was found between 16 mg/L and 32 mg/L ($P > 0.05$). So, combination treatment with 16 mg/L K-ras ASODN and 16 mg/L IGF-IR ASODN was employed, and the ASODNs were transfected into Patu8988 cells for 24, 48, 72 and 96 h. Patu8988 cell growth was inhibited at a significantly higher rate in the combination treatment than that in K-ras ASODN or IGF-IR ASODN alone at different transfection times ($P < 0.01$) (Figure 3C). The inhibition peak was reached at 48 h. Subsequently, the inhibition ability wore off, and the tumor cells recovered proliferation. Further experiments were conducted to assess the combined effects on the expression of K-ras or IGF-IR mRNA and protein, apoptosis, clone formation and tumor growth inhibition *in vivo* with the combination treatment of 16 mg/L K-ras ASODN and 16 mg/L IGF-IR ASODN at 37°C for 48 h.

Inhibition of colony formation by K-ras ASODN and IGF-IR ASODN alone or combination

Patu8988 cell proliferation treated in different groups was analyzed by soft agar colony formation assays. The average numbers of colonies in the control, K-ras ASODN, IGF-IR ASODN and combination group were 18.8, 11, 12 and 3, respectively. The Patu8988 cells in the combination groups formed significantly fewer colonies (6 fold decrease) in soft agar than those in the control groups did ($P < 0.05$ vs control). However, there were no statistical differences between K-ras ASODN groups and IGF-IR ASODN groups, although the number of colonies of IGF-IR ASODN groups was a little larger than those of K-ras ASODN groups ($P > 0.05$). At the same time, we noticed that the size of most of the colonies in the combination groups were much smaller than those in the control groups.

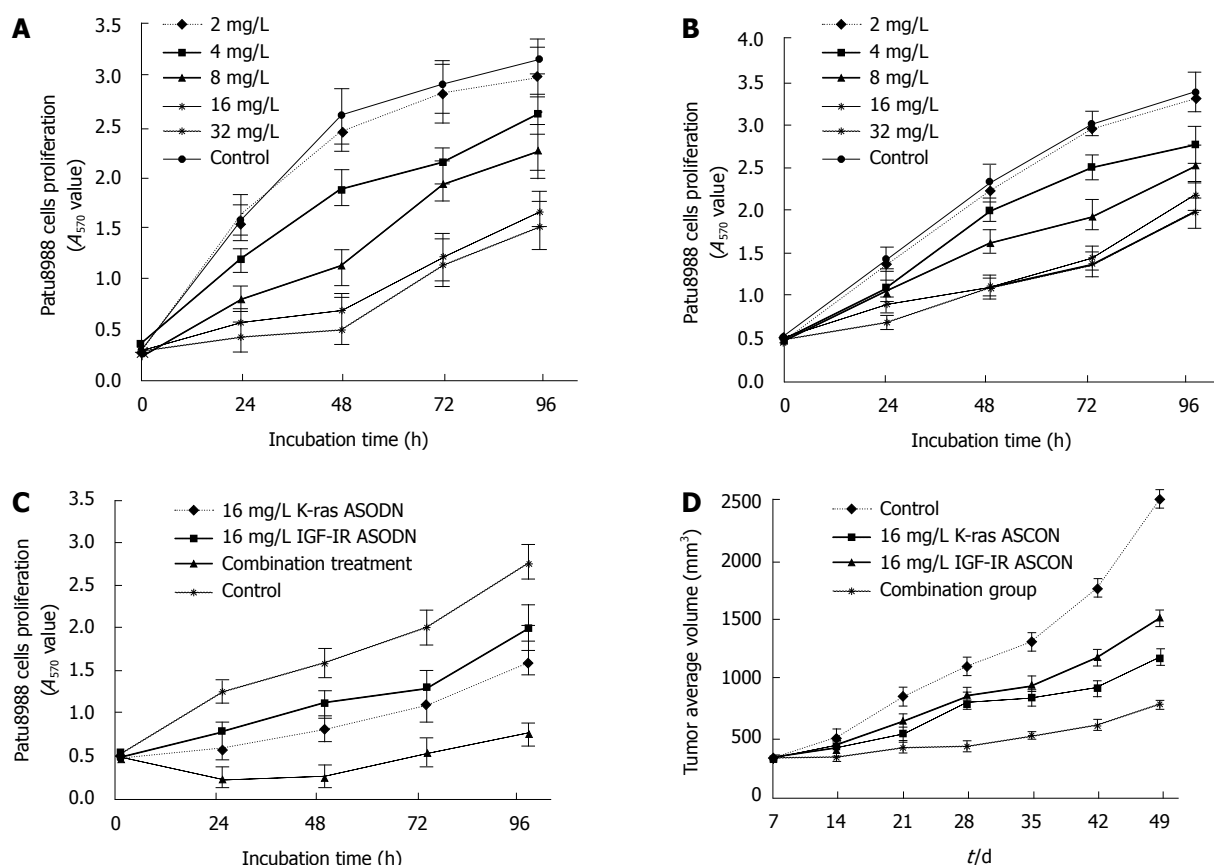


Figure 3 Each value represents the mean \pm SD from triplicate determinations. **A:** Growth curves of Patu8988 cells treated with different concentrations of K-ras ASODN at different incubation times; **B:** Growth curves of Patu8988 cells treated with different concentrations of IGF-IR ASODN at different incubation times; **C:** Growth curves of Patu8988 cells treated with the combination treatment of 16 mg/L K-ras ASODN and 16 mg/L IGF-IR ASODN at different incubation times; **D:** The inhibition effect of the combination treatment with K-ras ASODN and IGF-IR ASODN on tumor growth in nude mice.

Table 1 Protein expression for K-ras and IGF-IR on Patu8988 cells detected by flow cytometry (%)

Groups	Positive rate	
	K-ras protein	IGF-IR protein
K-ras ASODN	25.95 \pm 0.18 ^b	85.25 \pm 0.99
IGF-IR ASODN	69.18 \pm 0.87	40.78 \pm 1.42 ^b
Combination	19.69 \pm 1.15 ^b	38.25 \pm 1.22 ^b
Control	76.15 \pm 1.62	91.53 \pm 1.62

All values are presented as mean \pm SD of triplicate determinations. ^b $P < 0.01$ vs control group.

Apoptotic influence of Patu8988 cells treated with combination treatment of K-ras ASODN and IGF-IR ASODN

To further confirm the occurrence of apoptosis, we subjected the ASODNs-treated cells (48 h of ASODNs exposure) to annexin V-FITC/PI dual staining followed by flow cytometry analyses. The ratios of apoptosis cells were 21.54% \pm 0.93%, 12.76% \pm 0.74%, 8.43% \pm 0.51% and 1.60% \pm 0.19% in combination group, K-ras ASODN group, IGF-IR group and control group, respectively. Compared with the control group, statistically significant differences were observed ($P < 0.01$). The apoptotic rate of combination group was significantly higher than that of K-ras ASODN group alone or IGF-IR ASODN alone ($P < 0.05$). No

difference existed between K-ras ASODN group and IGF-IR group ($P > 0.05$) (Figure 4).

K-ras and IGF-IR protein expression of Patu8988 cells detected by flow cytometry

Flow cytometry, results showed that the positive rate of K-ras protein was 76.15% \pm 1.62% and 69.18% \pm 0.87% in control group and IGF-IR ASODN group, respectively. No statistical difference was found between the two groups ($P > 0.05$). But, in K-ras ASODN group and the combination group, K-ras protein was significantly decreased by 25.95% \pm 0.18% and 19.69% \pm 1.15%, respectively, compared with that of control group ($P < 0.01$). Flow cytometric analysis by using IGF-IR antibody showed that there was high expression in K-ras ASODN group, and control group with a positive rate of 91.53% \pm 1.62% and 85.25% \pm 0.99%, respectively. But, in IGF-IR ASODN, and combination group, IGF-IR protein expression was reduced to 40.78% \pm 1.42% and 38.25% \pm 1.22%, respectively. Significant differences were found when compared with control group ($P < 0.01$). All above results revealed that antisense oligodeoxynucleotides can inhibit corresponding protein expression. But, K-ras ASODN can not obviously inhibit the expression of IGF-IR protein, and IGF-IR ASODN can not obviously inhibit the expression of K-ras protein (Table 1).

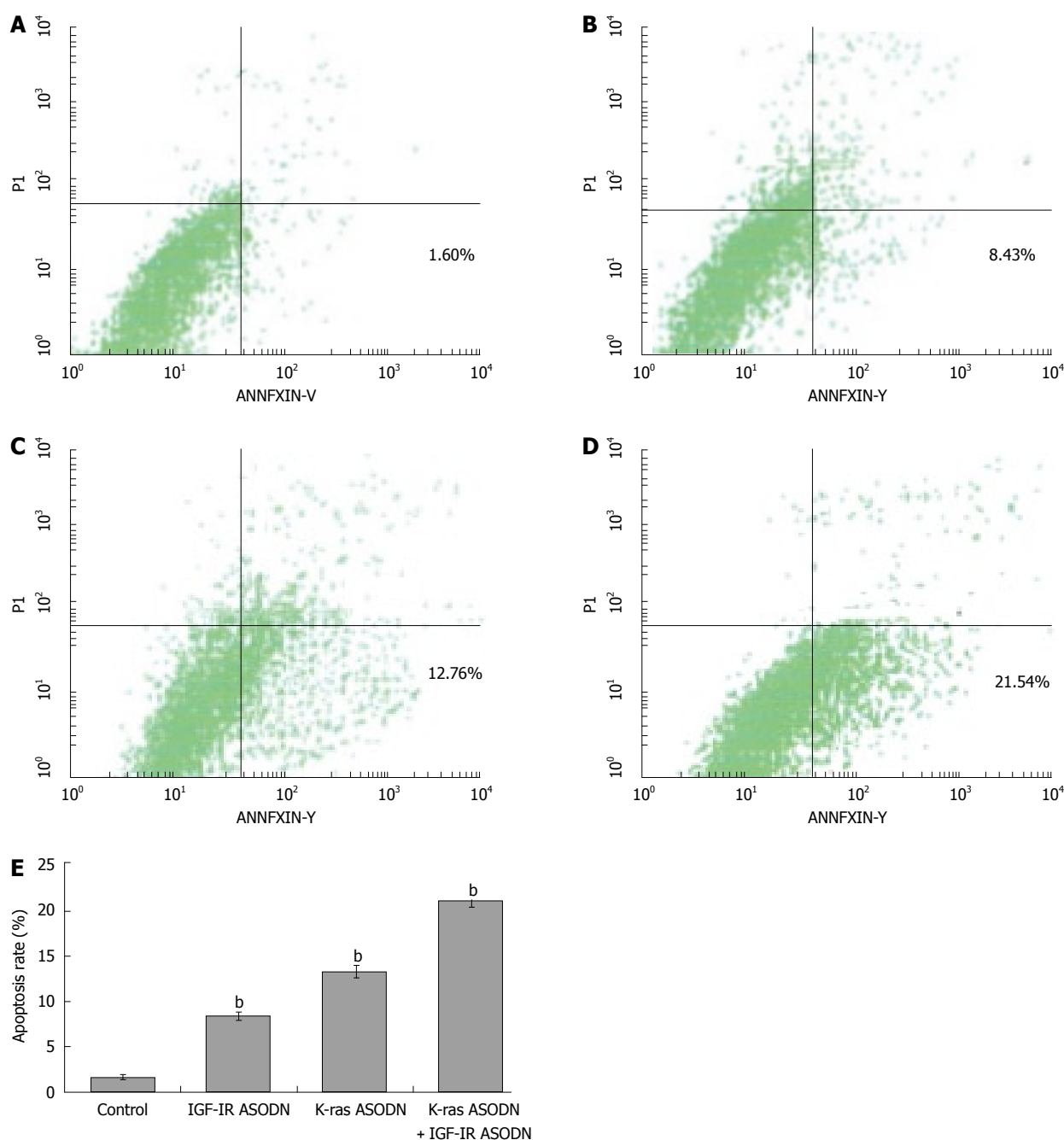


Figure 4 Apoptosis of Patu8988 cells detected by flow cytometry with Annexin V-FITC dual staining. **A:** Control group; **B:** IGF-IR ASODN group; **C:** K-ras ASODN group; **D:** K-ras ASODN + IGF-IR ASODN group; **E:** Apoptosis rate of Patu8988 cells for different groups. ^b $P < 0.01$, vs control group. Each value represents the mean \pm SD from triplicate determinations.

K-ras and IGF-IR mRNA expression of Patu8988 cells detected by RT-PCR

The mRNA expression intensities of *K-ras* gene and *IGF-IR* gene were analyzed by semiquantitative RT-PCR. The mRNA levels were normalized by internal control β -actin. At 48 h post-transfection, *K-ras* mRNA intensity levels were 0.389 ± 0.018 for IGF-IR ASODN group, 0.213 ± 0.027 for K-ras ASODN + IGF-IR ASODN group, 0.275 ± 0.023 for K-ras ASODN group and 0.391 ± 0.021 for control group. The statistical analysis showed that *K-ras* mRNAs of Patu8988 cells in K-ras ASODN group, and combination group were reduced significantly, compared with that of control group

($P < 0.05$). The inhibition rate reached 45.5% in the combination group. IGF-IR ASODN had no significant inhibitory effect on the expression of *K-ras* mRNA ($P > 0.05$, vs control) (Figure 5). As for *IGF-IR* gene, the relative mRNA levels were 0.642 ± 0.017 for *K-ras* ASODN, 0.355 ± 0.020 for the combination group, 0.387 ± 0.025 for IGF-IR ASODN group, and 0.630 ± 0.029 for control group. The statistical analysis showed that both IGF-IR ASODN group, and combination group could have a significant down-regulation effect on the mRNA expression of *IGF-IR* in Patu8988 cells ($P < 0.05$, vs control). The inhibition rate was 43.7% in the combination group. However, K-ras ASODN showed

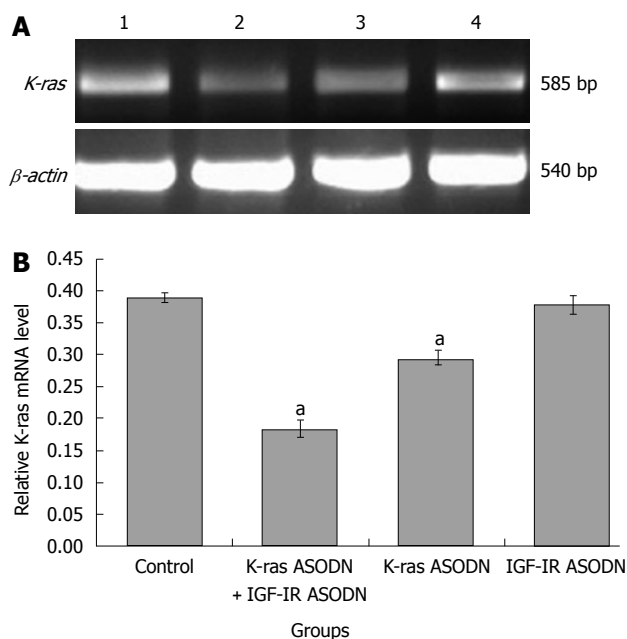


Figure 5 mRNA expression of *K-ras* gene in Patu8988 cells at 48 h post-transfection. **A:** RT-PCR analysis for *K-ras* gene in different groups. The β -actin gene was included as internal control. 1: IGF-IR ASODN group; 2: K-ras ASODN + IGF-IR ASODN; 3: K-ras ASODN group; 4: Control group; **B:** *K-ras* products quantified relative to the internal control β -actin. ^a $P < 0.05$, vs control. Each value represents the mean \pm SD from triplicate determinations.

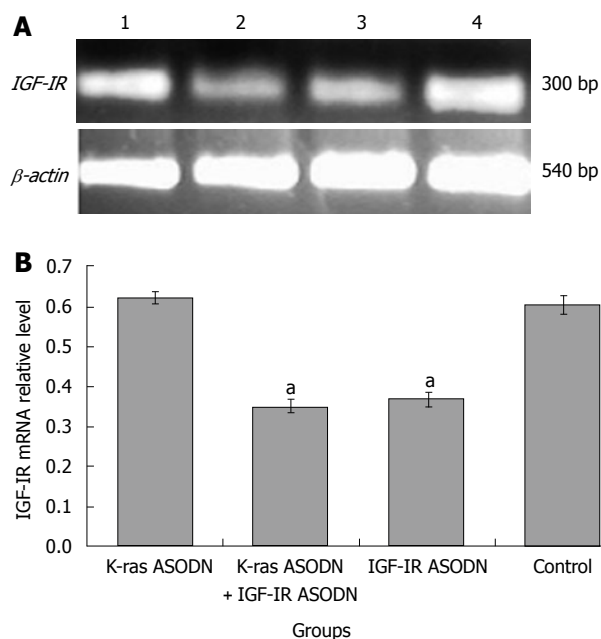


Figure 6 mRNA expression of *IGF-IR* gene in Patu8988 cells at 48 h post-transfection. **A:** RT-PCR analysis for *IGF-IR* gene in different groups. The β -actin gene was included as internal control. 1: K-ras ASODN group; 2: K-ras ASODN + IGF-IR ASODN; 3: IGF-IR ASODN group; 4: Control group; **B:** *IGF-IR* products quantified relative to the internal control β -actin. ^a $P < 0.05$ vs control. Each value represents the mean \pm SD from triplicate determinations.

no obvious inhibition for *IGF-IR* mRNA expression ($P > 0.05$ vs control) (Figure 6).

Inhibition of in vivo tumor growth by *K-ras* and *IGF-IR* downregulation

All nude mice were bearing pancreatic tumors from 7 to 10 d, and survived during the therapy with no red swelling, and disruption at the inoculation point. Before therapy, there were no significant difference for nude mice in weight and volume. As shown in Figure 3D, the tumor volume increased gradually in control group. The long diameter reached above 1.0 cm, and the volume reached $2230.0 \pm 65.6 \text{ mm}^3$ on the 49th d after inoculation. The tumor growth in K-ras ASODN group, IGF-IR ASODN group and combined group was inhibited with significant difference when compared with control group ($P < 0.01$). The therapeutic effect in the combined group was greater than that of K-ras ASODN or IGF-IR ASODN alone ($P < 0.01$). These results indicated that combination group exerted a strong growth-suppressive effect on pancreatic cancer. However, between K-ras ASODN and IGF-IR ASODN group, there was no obvious difference ($P > 0.05$). The results of immunohistochemical showed that K-ras and IGF-IR protein expression decreased in tumor tissues (data not shown).

Morphologic change under transmission electron microscopy

Using transmission electron microscope, we saw that the normal Patu8988 cells had intact cell membranes and nuclear membranes, distributed nuclear chromosomes,

distinct organelles, big nuclei and excessive nuclear division, which indicated that Patu8988 cells were highly malignant (Figure 7A). When Patu8988 cells were treated with K-ras ASODN combined with IGF-IR ASODN for 48 h, changes such as apoptosis, cell shrinkage, separation from neighboring cells, plasma condensation, plasma vacuolation, karyopyknosis, margination of condensed chromatin and membrane-bounded apoptotic bodies were observed (Figure 7B); some cells exhibited distinct deformation and disruption (Figure 7C).

DISCUSSION

The rapid development of molecular techniques has made it clear that tumorigenesis is actually a process of gene abnormalities. The strong invasiveness and rapid diffusive ability of pancreatic cancer are also closely associated with gene abnormalities. The study results of many years show that many genes' cooperation and many factors' participation contribute to the development of pancreatic cancer. Gene therapy brings hope for patients of pancreatic cancer. But, single gene therapy does not achieve ideal results. If two or more genes are combined to treat pancreatic cancer, in theory therapeutic effects will be better.

Since Almoguera *et al*^[17] first reported that K-ras mutation occurred in patients with pancreatic cancer, 85%-95% patients with pancreatic cancer have been found to have K-ras mutation, and most of these were point mutations at codon 12. Among those point mutations, GAT, GTT and CGT mutation styles comprised more than 95% of the point mutations at

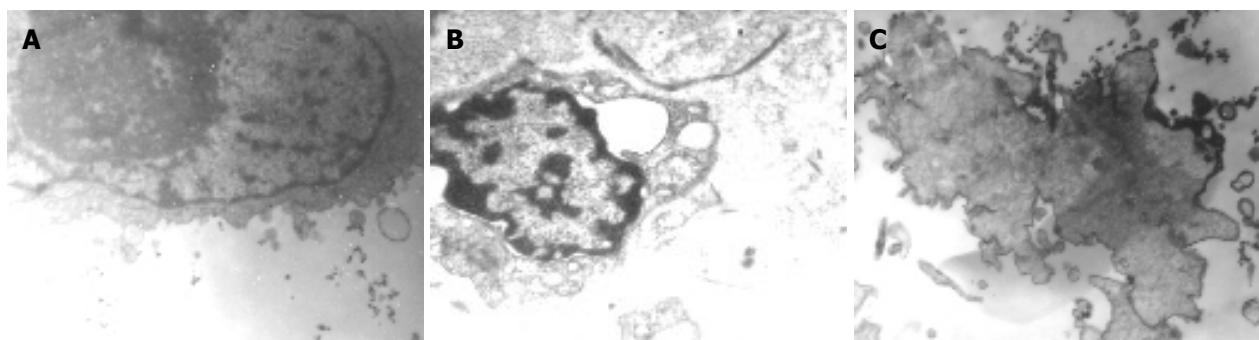


Figure 7 Transmission electron microscopic appearance of Patu8988 cell. **A:** Normal Patu8988 cell with intact cell membrane and nuclear membrane ($\times 12\,000$); **B:** Patu8988 cell treated with K-ras ASODN + IGF-IR ASODN for 48 h. The margination of condensed chromatin and membrane-bounded apoptotic bodies are observed ($\times 15\,000$); **C:** Necrotic cell ($\times 15\,000$).

codon 12^[18]. Therefore, K-ras point mutation at codon 12 is an early event for pancreatic cancer, which can be used as a target for early diagnosis and gene therapy^[19,20]. K-ras gene mutation destroys the GTP enzyme activity of ras protein and makes K-ras active constantly, which makes K-ras protein unable to block signals for growth. Recently, K-ras antisense oligodeoxynucleotides (ASODNs) have been transfected into pancreatic cells in China and abroad. Wang *et al.*^[21] first detected and confirmed K-ras gene mutation type CGT in human pancreatic carcinoma cell line PC-2. Then, K-ras mutation ASODN specific to CGT at codon 12 was used to explore its inhibitory effects on target gene in cell line PC-2. The results show that ASODN specific to K-ras point mutation CGT had significant inhibitory effects on target gene expression in human pancreatic carcinoma cells *in vitro*. Nakada *et al.*^[22] used ASODN specific to K-ras point mutation GAT at codon 12 to transfect into human pancreatic cancer cell line PANC-1 (with GAT mutation at codon 12), and the invasive activity was investigated using *in vitro* chemoinvasion assay. The results show that K-ras mutation ASODN specific to GAT at codon 12 strongly inhibited the invasive activity of the cell line PANC-1, but not in that with a wild type K-ras (BxPC-3). So, ASODNs specific to mutated K-ras genes can inhibit the proliferation and invasiveness of human pancreatic cancer cell lines. Specific antisense therapy to the point mutation of K-ras might be a new anticancer strategy for pancreatic cancer. However, these studies also indicate that adopting K-ras ASODN alone could not eradicate all the tumor cells. For exploring more effective therapy methods, some scholars abroad recently started to explore therapeutic alliance with diverse antisense oligodeoxynucleotides to treat pancreatic cancer, such as simultaneous transfection with mda-7 ASODN and K-ras ASODN into pancreatic cancer *in vitro* and *in vivo*. The results of their studies showed that the therapeutic effects of combination methods were better than that of one alone^[23,24].

IGF-IR is a receptor protein tyrosine kinase (RPTK) expressed in a wide variety of cell types including mesenchymal, epithelial, and hematopoietic cells. The receptor is a transmembrane heterotetramer consisting of two α -subunits and two β -subunits linked by

disulfide bonds. The binding of IGF-I to its receptor results in receptor oligomerization, activation of PTK, inter-molecular receptor autophosphorylation and phosphorylation of cellular substrates that consequently lead to gene activation, DNA synthesis and cell proliferation. Overexpression of IGF-IR stimulated cells not only to transform toward malignance and sustain malignant phenotype, but also to promote tumor cells' anti-apoptosis, mitosis, proliferation and invasiveness. Min *et al.*^[10] reported that IGF-IR overexpressed in pancreatic cancer and down-regulation of IGF-IR expression using monoclonal antibodies or antisense oligodeoxynucleotides could inhibit tumor cell growth both *in vitro* and *in vivo*^[25-27]. However, the inhibition ratio was not high^[24].

Considering the important effects of K-ras gene point mutation at codon 12 in pancreatic cancer and the broad tumorigenesis of IGF-IR gene, our study explored the effects of Patu8988 cell proliferation, apoptosis and target gene expression using combined antisenses with K-ras ASODN against K-ras point mutation at codon 12 and IGF-IR ASODN against insulin-like growth factor-1. We noticed that different doses for K-ras ASODN or IGF-IR ASODN could inhibit Patu8988 cell growth. But, combinations could produce greater effects ($P < 0.01$). The results were also confirmed in animal experiments. Compared with the single method, combination could obviously induce Patu8988 cell apoptosis, and reduce protein and mRNA expression of K-ras and IGF-IR. In our study, the inhibition effects of K-ras ASODN were better than that of IGF-IR ASODN. Ras protein is not only one of the signal pathways of IGF-IR, but also the pathway for many other growth factors, such as VEGF. Therefore, mutated ras protein not only amplifies the IGF-IR signal, but also amplifies signals for many other growth factors to inhibit cell apoptosis, and also induces vascular growth and cell proliferation. IGF-IR ASODN can inhibit only IGF-IR signal. But, K-ras ASODN can inhibit signals for many factors. Therefore, the inhibition effects of K-ras ASODN was better than that of IGF-IR ASODN. So, combined therapy can inhibit signals on two sides. After being treated with K-ras ASODN and IGF-IR ASODN together, some cells appeared in the

form of apoptosis, some others in the shape of edema or deformation, which indicated that antisense not only induced apoptosis, but also promoted cell death directly.

Our study shows that K-ras ASODN combined with IGF-ASODN obviously inhibited Patu8988 cell growth, and induced cell apoptosis and death. The mechanism may be associated with the inhibition of mRNA, and protein expression of K-ras and IGF-IR in Patu8988 cells. Cooperation with two synergistic antisense oligodeoxynucleotides could provide a new gene therapeutic strategy against pancreatic cancer. Meanwhile, the results of our study show that K-ras ASODN and IGF-IR ASODN inhibited tumor growth alone or in combination. However, a rapid cell proliferation tendency was seen in later stage of combined therapy. We speculated that this phenomenon might be associated with the degradation of ASODN in the late stage of treatment. We need to do further study to learn the relationship between dose-effect and time-effect. On the other hand, the development of pancreatic cancer involves many genes; we can not inhibit tumor growth completely by suppressing two genes of *K-ras* and *IGF-IR*, only partial.

COMMENTS

Background

Pancreatic carcinoma is the cancer that has the highest *K-ras* gene mutation rate. 95% of mutations happen at codon 12. Three major mutation types have been reported, including CGT, GAT and GTT. Antisense oligodeoxynucleotides (ASODNs) specific to CGT and GAT point mutations in human pancreatic cancer cell lines were reported; the ASODN against GTT point mutation in pancreatic cancer remains unclear. Some studies reported that type 1 insulin-like growth factor receptor (IGF-IR) is overexpressed in pancreatic cancer and down-regulation of IGF-IR expression using ASODNs could inhibit tumor cell growth. In this article, whether K-ras ASODN specific to GTT mutation in alliance with IGF-IR ASODN regulate Patu8988 proliferation, apoptosis, target gene expression *in vitro* and *in vivo* was investigated.

Research frontiers

In previous studies, antisense oligodeoxynucleotides (ASODNs) specific to CGT and GAT point mutation of *K-ras* gene were demonstrated to inhibit proliferation in pancreatic cancer.

Innovations and breakthroughs

It was found in the present study that K-ras ASODN combined with IGF-IR ASODN could cooperatively inhibit the growth of Patu8988 cells and induce their apoptosis via reinforcing specific down regulation of *K-ras* and *IGF-IR* mRNA and protein expression.

Applications

Cooperation with two synergistic antisense oligodeoxynucleotides could provide a new gene therapeutic strategy against pancreatic cancer.

Terminology

PCR-SSP is polymerase chain reaction with special sequence primers; *K-ras* ASODN is an antisense oligodeoxynucleotide against *K-ras* gene; IGF-IR ASODN is an antisense oligodeoxynucleotide against *IGF-IR* gene; GAT, CGT and GGT are three major point mutation types at codon 12 of *K-ras* gene.

Peer review

This is an interesting study that identifies molecular pathways that may be therapeutically targeted to inhibit pancreatic cancer growth.

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BASIC RESEARCH

Phosphatidylinositol 3-kinase/Akt pathway regulates hepatic stellate cell apoptosis

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Abstract

AIM: To investigate the role of phosphatidylinositol 3-kinase (PI 3-K)/Akt signaling pathway in the balance of HSC activation and apoptosis in rat hepatic stellate cells (HSC).

METHODS: An activated HSC cell line was used in this study. LY 294002, the PI 3-K/Akt signal pathway blocker was used to investigate the molecular events on apoptosis in HSC and to interpret the role of this pathway in HSC apoptosis. Immunocytochemistry, Western blot and reverse transcription polymerase chain reaction (RT-PCR) analysis were applied to detect the expression of PI 3-K, and simultaneously phosphorylated-Akt (p-Akt) and total-Akt were determined by Western blot. The HSC apoptosis was examined by annexin-V/propidium iodide double-labelled flow cytometry and transmission electron microscopy.

RESULTS: The apoptosis rates in LY 294002 (30.82% \pm 2.90%) and LY 294002 + PDGF-BB (28.16% \pm 2.58%) groups were significantly increased compared with those of control (9.02% \pm 1.81%) and PDGF-BB (4.35% \pm 1.18%). PDGF-BB augmented PI 3-K and p-Akt expression. LY 294002 significantly reduced the contents of PI 3-K and p-Akt. mRNA transcription evaluated by RT-PCR showed similar tendencies as protein expression.

CONCLUSION: Inhibition of PI 3-K/Akt signaling pathway induces apoptosis in HSC.

INTRODUCTION

The activation and proliferation of hepatic stellate cells (HSC) is a key event in fibrogenesis. On the other hand, HSC apoptosis results in fibrolysis and fibrotic regression^[1-3]. Therefore, by understanding pro- and anti-apoptogenic factors, new therapeutic targets will be identified for the treatment of liver fibrosis. However, intracellular signals that regulate HSC apoptosis are still obscure. Our previous study has demonstrated that a cross talk between platelet-derived growth factor (PDGF), the most potent proliferative cytokines for HSC, and focal adhesive kinase (FAK), a nonreceptor tyrosine kinase, is involved in an integrin signaling pathway^[4-6]. This interaction is essential for PDGF to induce HSC proliferation^[7].

Phosphatidylinositol 3-kinase (PI 3-K)/Akt signaling molecules, which are downstream of FAK, are also activated by PDGF. The activated PI 3-K/Akt participate in regulation of HSC migration, proliferation, collagen secretion and adhesion^[5]. PI 3-K is involved in regulating a number of cellular responses, such as cell growth, survival and migration. Akt, a serine/threonine kinase, is downstream of PI 3-K and an important anti-apoptotic factor. The purpose of the present study was to determine the effects and the molecular mechanisms whereby PI 3K/Akt influence apoptosis in HSC.

MATERIALS AND METHODS

Reagents and instruments

RPMI 1640 culture medium was obtained from Gibco (Invitrogen Corporation Carlsbad, California USA), fetal

bovine serum (FBS) from Sijiqing Company of Bio-products (Hangzhou, China) and LY 294002 from Sigma (Saint Louis, Missouri USA). Rat PDGF-BB was from Cytolab Biochemicals (Rehovot, Israel USA). Rabbit anti-phospho-Ser⁴⁷³ Akt polyclonal antibody and rabbit anti-PI 3-K p85 α polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-total Akt polyclonal antibody was purchased from Cell Signaling Biotechnology (Beverly, MA USA). RT-PCR amplification system was from Promega (Madison, WI USA). Annexin-V/Propidium iodide double-labelled flow cytometry kit was purchased from Baosai Company of Bio-products (Beijing, China). CO₂ incubator was from SANYO Company (Chatsworth, CA Japan). Flow cytometer type FACS-420 was purchased from Becton Dickinson Company (Franklin Lakes, NJ USA).

Cell line and cell culture

The activated HSC phenotype, from CCl₄ induced cirrhotic liver, was provided by Professor Greenwel (USA). This cell line is similar to that of primary cultured HSC except for absent expression of collagen type IV^[8]. HSC was cultured in RPMI 1640 media supplemented with 10% FBS, penicillin (100 IU/mL)/streptomycin (100 μ g/mL), and glutamine (4 mmol/L) in a 95% air and 5% CO₂ humidified atmosphere. Cell viability was determined by trypan blue exclusion staining. When exponential growing HSC populations were found to be nearly 100% confluence, HSC (2×10^5 /mL) was incubated in serum free media for 24 h and then split into four groups: control, PDGF-BB (PDGF 20 ng/mL), PDGF-BB + LY 294002 group (LY 294002 50 μ mol/L, PDGF-BB 20 ng/mL) and LY 294002 (LY 294002 50 μ mol/L). Activated HSC was serum-starved for 24 h, and then treated as described above. One set of the cells was harvested for 4 h later for mRNA transcription and protein expression assay. The same set of cells was harvested 20 h later for electron microscopic examination, flow cytometric analysis and immunostaining.

Electron microscopic study

The cultured HSC of the four groups were collected, digested, washed with phosphate buffered solution (PBS), fixed with 4% glutaraldehyde for 2 h, and then fixed with osmium tetroxide for 1 h, stained with uranium acetate, embedded into 6.8[#] epoxide resin, after sectioning into ultra-thin slices, the cells were stained with lead citrate and examined under transmission electron microscopy.

Flow cytometric analysis

Four groups of HSC were collected, washed with pre-cooled PBS twice, fixed with cold 70% ethanol, digested with 50 μ g/mL RNase at 37°C, and stained with 65 μ g/mL propidium iodide at 4°C for 1 h, and then the flow cytometric analysis was conducted. 10^4 cells were used in apoptotic analyses. Avian red blood cells were used as control. The variation coefficient (CV) of the assay was less than three percent. The data collected were analyzed in a computer (type HP-300

Consort 30), and analyzed with Histogram Statistics software.

RT-PCR

Total RNA from cultured HSC was extracted by the Trizol method. After RT (41°C, 45 min) and pre-denaturation (94°C, 2 min), PCR amplification was carried out for 35 cycles. The primers, as designed according to GenBank, were as follows: PI 3-K p85: sense 5'-CCCAGGAGCGGTACAGCAAAGAA-3', antisense 5'-TGGGGCAAATCCTCATCATCTTC-3'; β -actin: sense 5'-ACAGAGTACTTGCGCTCAGGAG-3', antisense 5'-GTCACCCACACTGTGCCCATCT-3'. The initial reaction mixture contained 1 μ mol/L primers, AMV reverse transcriptase, RNA Tfl DNA polymerase and 25 mmol/L MgSO₄. Total volume was 50 μ L. The amplicon lengths of the PCR products were: PI 3-K p85 355 bp and β -actin 585 bp. Denaturation (94°C for 40 s), annealing (52°C for 1 min), and extension (72°C for 1.5 min). PCR products were analyzed by electrophoresis in a 1.5% agarose gel. β -actin amplification was used as internal control.

Immunostaining

Cultured cells were seeded onto glass coverslips and allowed to adhere for 20 h under routine culture conditions. After incubation, cells were washed twice with cold PBS, fixed and permeabilized by cold methanol for 8 min, and coverslips were then air-dried at room temperature. Immunostaining was performed using the SP kit according to the instructions of the manufacturer. Briefly, HSC was washed with washing buffer (0.2% Tween PBS), incubated with peroxidase-blocking reagent and normal goat serum. After rinsing, coverslips were incubated with primary antibodies (rabbit anti-phospho-Ser⁴⁷³, Akt polyclonal antibody (1:100) and rabbit anti-PI 3-K p85 α polyclonal antibody (1:50) at room temperature for 12 h. After rinsing primary antibody with washing buffer, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was added, and incubated at 37°C for 30 min. Following rinsing with washing buffer, 3,3'-diamino benzidine/enhancer (DAB) solution was added and incubated at room temperature for 5 min. After washing, the coverslips were counterstained with hematoxylin for 1 min, rinsed with water and dried for 10 min. The slides were dehydrated with 100% ethanol for 20 s twice, and finally with xylene for 20 s. The coverslips were mounted onto glass slices and viewed under microscopy.

Western blot analysis of PI 3-K, Akt and p-Akt

Cultured HSC was washed with PBS and lysed with protein sample buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L phenylmethyl sulfonylfluoride, and 2 mg/L leupeptin). The protein concentrations were measured using Coomassie brilliant blue chromatometry. Equal amounts (100 μ g) of the denatured proteins per lane were loaded and separated on sodium dodecyl sulfate-10% polyacrylamide gels for p-Akt (Ser⁴⁷³), Akt and β -actin, and 8% SDS-polyacrylamide gels for PI 3-K. After electrophoresis, proteins were

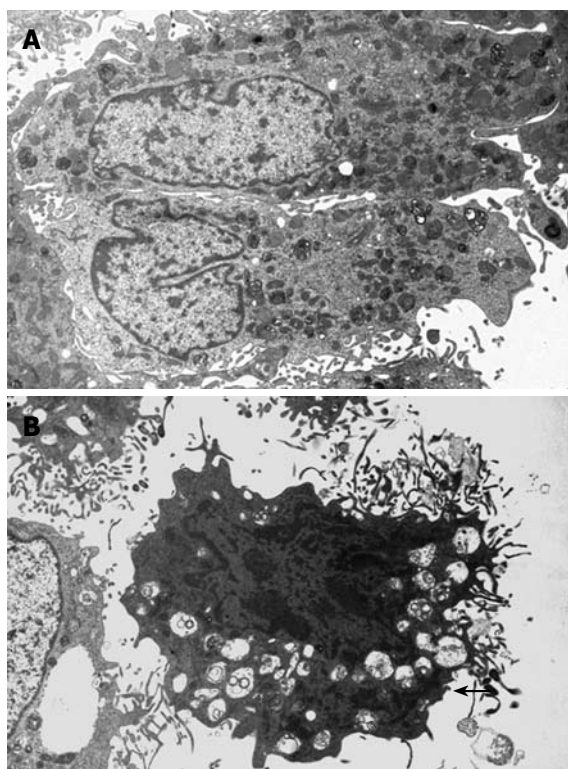


Figure 1 Transmission electron micrography of cultured HSC. **A:** Control HSC showing nuclear is intact and the mitochondria is smooth; **B:** LY 294002 treated HSC: Chromatins condensed, shrunken and aggregated along inside the nuclear membrane. The arrows points at the apoptotic bodies ($\times 5000$).

transferred to nitrocellulose. The membranes were blocked for 12 h with 5% powdered skim milk in Tris-HCl-buffered saline containing 0.05% Tween 20 (TBS-T). After washed with TBS-T for three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:3000). After washing 5 times, the blots were detected with the enhanced chemiluminescence method (ECL) assay.

Statistical analysis

Results were expressed as mean \pm SD. Statistical analysis was performed by one-way ANOVA, and, when the *F* value was significant, by Student-Newman-Keuls test. *P* value less than 0.05 was considered statistically significant.

RESULTS

LY 294002 induces apoptosis in rat HSC

Under the transmission electron microscopy (Figure 1), the cells in LY 294002 and LY 294002 + PDGF-BB groups showed condensed chromatin, shrunken and aggregated along inside the nuclear membrane. The morphology of the cells showed spherical, petal or crescent shape, apoptotic bodies were found in some cells, while in control and PDGF-BB groups, HSC revealed normal silhouettes.

As listed in Table 1, the apoptotic rates in LY 294002 and LY 294002 + PDGF-BB groups were significantly increased compared with that of control group (30.82%

Table 1 Apoptosis induction of PDGF-activated HSC by LY 294002 (mean \pm SD)

Groups	Apoptotic rate of HSC
Control	9.02 \pm 1.81
PDGF-BB	4.35 \pm 1.18 ^a
PDGF-BB + LY 294002	28.16 \pm 2.58 ^b
LY 294002	30.82 \pm 2.90 ^b

^a*P* = 0.005 vs control group; ^b*P* < 0.0001 vs control group.

Table 2 Protein expression of PI3K and p-Akt by immunocytochemistry (mean \pm SD)

Groups	p-Akt (%)	PI3K (%)
Control	33.65 \pm 1.90	35.82 \pm 1.87
PDGF-BB	58.86 \pm 1.89 ^a	60.85 \pm 1.75 ^a
LY294002 + PDGF-BB	24.24 \pm 0.84 ^a	25.18 \pm 1.05 ^a
LY294002	23.65 \pm 1.90 ^a	23.63 \pm 1.64 ^a

^a*P* < 0.0001 vs control group.

\pm 2.90%, 28.16% \pm 2.58% and 9.02% \pm 1.81%, respectively; *P* < 0.01). There was no significant difference between LY 294002 and LY 294002 + PDGF-BB groups (*P* = 0.12).

PDGF-induced HSC activation abolished by LY 294002

PI 3-K assay: PDGF-BB could significantly increase PI 3-K expression in rat HSC. LY 294002 not only decreased the PI 3-K positive cells in control group, but also reduced PI 3-K content in PDGF-BB activated cells (Figure 2 and Table 2). These immunocytochemical results were supported by Western blots: the band density in PDGF-BB group was the strongest. LY 294002 not only decreased the PI 3-K expression in control cells, but also decreased the protein content in PDGF-BB activated cells (Figure 3). The effects of LY 294002 were reflected not only by the protein expression levels, but also by mRNA transcription (Figure 4).

Akt assay: Immunocytochemistry showed that p-Akt expression was parallel with PI 3-K: PDGF-BB stimulated p-Akt protein expression, while LY 294002 not only inhibited p-Akt concentration in control group, but also abolished PDGF stimulated p-Akt expression (Figure 2 and Table 2). Western blots showed the same pattern as immunocytochemistry for p-Akt. Noticeably, the total Akt levels assessed by Western blotting revealed no change in all four groups (Figure 5).

All the results above showed that PDGF activated the whole PI 3-K/Akt/p-Akt, pathway, while LY 294002 decreased the entire pathway activity not only in control cells, but also in PDGF stimulated circumstances.

DISCUSSION

It is activation of HSC that initiates liver fibrosis, and regardless of the etiology, the final pathway is to activate HSC. The activated HSC is proliferative, fibrogenic,

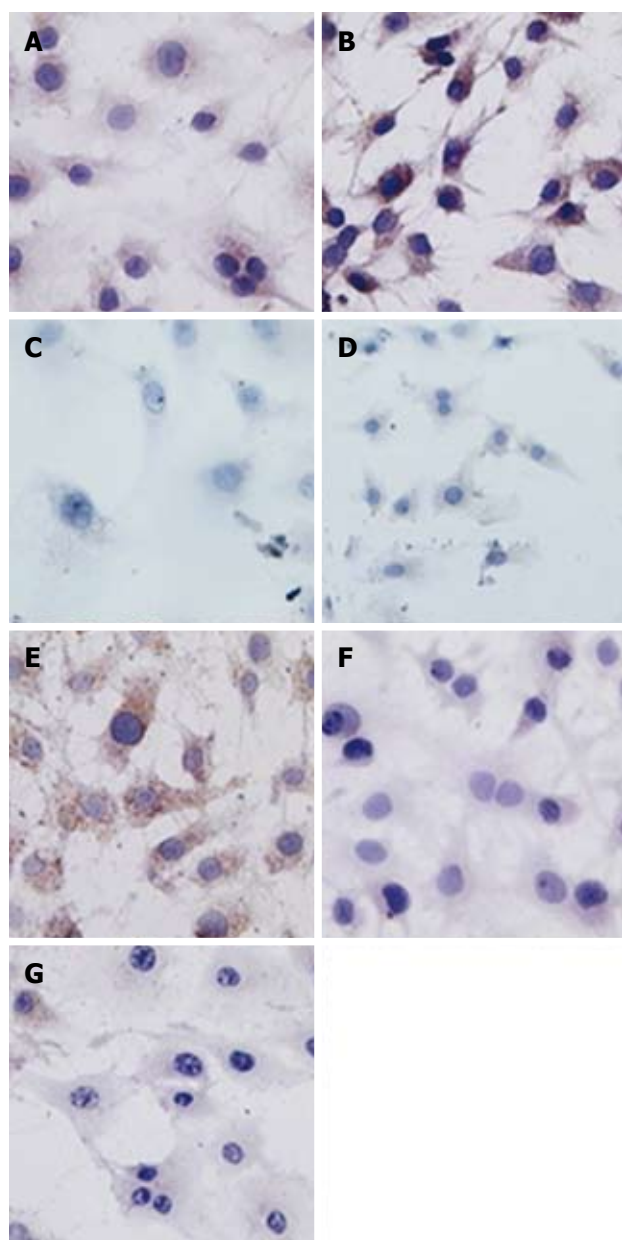


Figure 2 Immunocytochemistry ($\times 400$). **A:** In negative control, the primary antibody was omitted; **B** and **E:** PI 3-K p85 and p-Akt⁴⁷³ staining in the PDGF-BB group; **C** and **F:** PI 3-K p85 and p-Akt⁴⁷³ expression in the PDGF-BB and LY 294002 groups; **D** and **G:** PI 3-K p85 and p-Akt⁴⁷³ staining in the LY 294002 group. Rabbit anti-PI 3-K p85 α polyclonal antibody (1:50) and rabbit anti-phospho-Ser⁴⁷³ Akt polyclonal antibody (1:100) used as primary antibodies.

and contractile myofibroblasts^[5,9]. The proliferation and apoptosis of HSC keep balance in physiological situation. Once this equilibrium is broken under chronic injury, continuing HSC proliferation and collagen secretion will cause fibrosis^[10]. Therefore, inhibiting the proliferation of HSC and inducing its apoptosis are two ways to delay or stop fibrogenesis. PI 3-K and Akt are two molecules that stimulate HSC proliferation^[1,2].

PI 3-K is comprised of an 85-kDa regulatory subunit, and a catalytic 110-kDa subunit. Activated by growth factors such as PDGF and insulin-like growth factor (IGF), PI 3-K catalyzes the phosphorylation of phosphatidylinositol (PI) at the 3-OH position to generate phosphatidylinositol triphosphate (PIP3). Activated

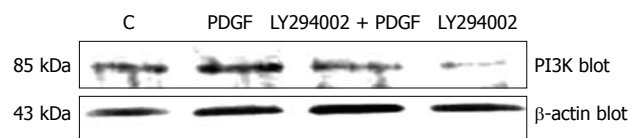


Figure 3 Representative Western blot analysis of PI 3-K protein expression in HSC with β -actin as internal control. From left, 1st lane, control HSC; 2nd lane, PDGF stimulated HSC; 3rd lane, PDGF + LY 294002 group and 4th lane, LY 294002 treated HSC.

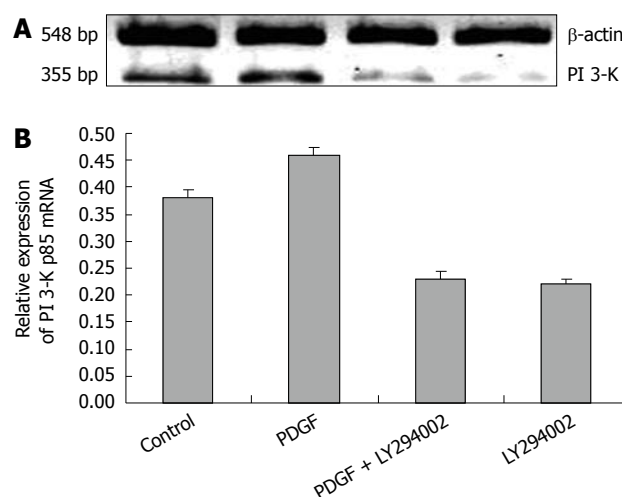


Figure 4 Representative RT-PCR photography of PI 3-K mRNA transcription from rat HSC, β -actin as internal control. **A:** From left, 1st lane, control HSC; 2nd lane, PDGF stimulated HSC; 3rd lane, PDGF + LY 294002 group and 4th lane, LY 294002 treated HSC. **B:** A graphic analysis of the RNase protection assay.

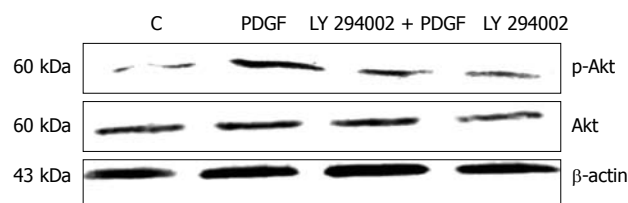


Figure 5 Representative Western blot analysis of p-Akt and total Akt protein expressions in HSC, β -actin as internal control. From left, 1st lane, control HSC; 2nd lane, PDGF stimulated HSC; 3rd lane, PDGF + LY 294002 group and 4th lane, LY 294002 treated HSC.

growth factor receptor migrates to the cellular membrane, and this results in PI 3-K activation, that later combines with its substrates. Akt, a serine/threonine kinase, is also known as protein kinase B (PKB). Three isoforms have been identified thus far in the mammalian cells: Akt1/PKB α , Akt2/PKB β , and Akt3/PKB γ . The three isoforms have similar phosphorylation sites. One is in the N-terminal Thr³⁰⁸. And the other in the COOH-terminal hydrophobic motif Ser⁴⁷³. The phosphorylation of both sites is necessary for full activity. Activated PI 3-K/Akt signaling inhibits apoptosis, and stimulates cell proliferation, migration, survival as well as other biological behaviours^[11].

Among the three PDGF isoforms, PDGF-AA, PDGF-BB and PDGF-AB, PDGF-BB has been identified as the most potent mitogen for HSC^[7,12]. Liver

fibrosis is associated with an increase in PDGF protein expression and increased PDGF receptor expression. PDGF receptors have intrinsic tyrosine kinase activity and upon binding to its ligand, PDGF receptors can phosphorylate itself at tyrosine residues. In cultured human HSC, PDGF can activate PI 3-K which is necessary for both mitogenesis and chemotaxis^[13-15]. This pathway is up-regulated during liver injury *in vivo*. Wortmannin and LY 294002, two specific PI 3-K inhibitors, can both dose-dependently block PI 3-K activity induced by PDGF and inhibit DNA synthesis^[16].

Our immunocytochemistry in the present study found that PI 3-K and p-Akt were mainly in HSC cytoplasm. Immunocytochemistry, Western blot and RT-PCR demonstrated that PDGF-BB can significantly stimulate PI 3-K and p-Akt productions in both mRNA transcription (PI 3-K) and protein expression levels, LY 294002 not only reduced PI 3-K and p-Akt contents in control group, but also reduced PDGF stimulated PI 3-K expression and Akt phosphorylation. There was no significant difference between the groups of LY 294002 only and LY 294002 + PDGF. This means that LY 294002 can abolish the stimulatory effect of PDGF. Our results also showed that there was no significant difference of total-Akt levels among four groups, indicating that instead of total Akt syntheses, LY 294002 mainly inhibits Akt phosphorylation. This *in vitro* study is consistent with the well-documented data which confirmed Akt phosphorylation being inhibited by LY 294002 in bile duct-ligated (BDL) animals. In addition, PDGF can induce Akt phosphorylation and LY 294002 blocks this phosphorylation.

The Akt family represents pivotal factors to promote cell survival, proliferation and inhibit apoptosis. Akt plays an important role in inhibiting apoptosis in variety of cells such as uterine leiomyoma cell lines, hematopoietic progenitor/stem cell lines, pancreatic beta cells and islet beta cells *etc*^[17-20]. Our results showed that Akt also inhibited apoptosis in HSC. Following PI 3-K/Akt pathway inhibition, HSC revealed typical apoptotic morphous under transmission electron microscopy. Microvilli on the cell surface decreased, became short, and even disappeared. Cells shrank, cytoplasm condensed, ribosome and mitochondria aggregated. The chromatin condensed and shrank and aggregated along inside of the nuclear membrane to form of balls, petals and crescents. Sometimes, apoptotic bodies formed. The apoptosis rates of both LY 294002 treated groups were significantly increased compared with that of the control group and the PDGF group and thus, we conclude that PI 3-K/Akt inhibition enhances HSC apoptosis.

In summary, the data obtained have shown that inhibition of PI 3-K/Akt signaling pathway can induce apoptosis in rat HSC, even under strong mitogen stimulator (PDGF). The activation of HSC is essential for fibrogenesis, and PI 3-K/Akt signaling is indispensable to support HSC activation. Therefore, blocking the PI 3-K/Akt signaling pathway may provide a potential therapeutic benefit for liver fibrosis.

COMMENTS

Background

Hepatic fibrosis represents a reversible and dynamic process in response to a variety of chronic stimuli. Activation of the hepatic stellate cell (HSC), a perisinusoidal cell that resides in the liver in a quiescent state, is responsible for the increased synthesis and deposition of extracellular matrix (ECM) in the liver, and plays a critical role in fibrogenesis. The paradigms of HSC activation and apoptosis remain valuable frameworks for understanding pathways of hepatic fibrogenesis and fibrosis regression. HSC apoptosis results in fibrolysis and fibrotic regression. Therefore, by understanding the pro- and anti-apoptogenic factors, new therapeutic targets will be identified for the treatment of liver fibrosis. Phosphatidylinositol 3-kinase (PI 3-K)/Akt are components of the major intracellular signaling pathways elicited by platelet-derived growth factor (PDGF) in HSC. Intracellular signals that regulate HSC apoptosis are still obscure. Our previous study has demonstrated that a cross talk between PDGF, the most potent proliferative cytokines for the HSC, and focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, is involved in integrin signaling pathway. This interaction is essential for PDGF to induce HSC proliferation.

Research frontiers

The activated HSC is primarily responsible for excessive deposition of ECM proteins and collagen deposition during liver fibrosis. Substantial insight is being gained into the molecular mechanisms responsible for apoptosis in the HSC. The activated HSC becomes responsive to both proliferative and fibrogenic cytokines. These cytokines activate both mitogen-activated protein kinase (MAPK) signaling, involving p38, and FAK-PI 3-K-Akt-p70 (S6K) signaling cascades. PI 3-K/Akt constitutes an important pathway regulating the signaling of multiple biological processes such as apoptosis, metabolism, cell proliferation and cell growth. Activating mutations, which have been reported for PI 3-K and Akt in tumors, are able to confer tumorigenic properties in several cellular systems. These regulate the proliferative response, activating cell cycle progression as well as collagen gene expression. It is anticipated that by understanding the molecular mechanisms responsible for HSC proliferation, apoptosis, and excess ECM production new therapeutic targets will be identified for the treatment of liver fibrosis.

Innovations and breakthroughs

HSC apoptosis results in fibrolysis and further on, fibrotic regression. Intracellular signals that regulate HSC apoptosis are still obscure. Our previous study has demonstrated that a cross talk between PDGF and FAK is involved in integrin signaling pathway. PI 3-K/Akt signaling molecules, which are downstream of FAK, are also activated by PDGF. Up to now, few studies have addressed the function of PI 3-K/Akt signal transduction pathways on the apoptosis of HSC. The present study determined the effects and the molecular mechanisms whereby PI 3-K/Akt influences apoptosis in HSC, and showed that PDGF activated the whole PI 3-K/Akt/p-Akt, pathway, while LY 294002 decreased the entire pathway activity not only in control cells, but also in PDGF stimulated circumstances. PI 3-K/Akt signal molecules will be new therapeutic targets for the treatment of liver fibrosis.

Applications

The present study has shown that inhibition of PI 3-K/Akt signaling pathway can induce apoptosis in rat HSC, even under strong mitogen stimulators (PDGF). The activation of HSC is essential for fibrogenesis, and PI 3-K/Akt signaling is indispensable to support HSC activation. Therefore, blocking the PI 3-K/Akt signaling pathway may provide a potential therapeutic benefit for liver fibrosis.

Peer review

The paper by Wang *et al* presents an interesting study of the role of PI 3-K/Akt in controlling apoptosis of hepatic stellate cells. The purpose of the present work was to determine whether inhibition of PI 3-K affected apoptosis in HSC. The methods used to measure expression of PI 3-K and phosphorylation of Akt as well as total Akt were acceptable, and the assessment of apoptosis in HSC also reliable. The results very clearly show that the apoptotic rate was markedly increased in presence of LY294002.

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RAPID COMMUNICATION

Ketamine anesthesia reduces intestinal ischemia/reperfusion injury in rats

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showed significantly less injury in rats that received ketamine than in rats that did not (2.35 ± 1.14 vs 4.58 ± 0.50 , $P < 0.0001$). The distance traveled by a marker, expressed as percentage of total intestinal length, in rats that received pentobarbital sodium was $20\% \pm 2\%$ in comparison with $25.9\% \pm 1.64\%$ in rats that received ketamine ($P = 0.017$). BER was not statistically different between groups.

CONCLUSION: Our results show that ketamine anesthesia is associated with diminished intestinal injury and abolishes the intestinal transit delay induced by ischemia/reperfusion.

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Key words: Ischemia/reperfusion; Ketamine; N-methyl-D-aspartate; Intestinal motility; Tissue damage

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Abstract

AIM: To investigate the effects of ketamine anesthesia on the motility alterations and tissue injury caused by ischemia/reperfusion in rats.

METHODS: Thirty male Wistar rats weighing 200-250 g were used. Ischemia was induced by obstructing blood flow in 25% of the total small intestinal length (ileum) with a vascular clamp for 45 min, after which either 60 min or 24 h of reperfusion was allowed. Rats were either anesthetized with pentobarbital sodium (50 mg/kg) or ketamine (100 mg/kg). Control groups received sham surgery. After 60 min of reperfusion, the intestine was examined for morphological alterations, and after 24 h intestinal basic electrical rhythm (BER) frequency was calculated, and intestinal transit determined in all groups.

RESULTS: The intestinal mucosa in rats that were anesthetized with ketamine showed moderate alterations such as epithelial lifting, while ulceration and hemorrhage was observed in rats that received pentobarbital sodium after 60 min of reperfusion. Quantitative analysis of structural damage using the Chiu scale

INTRODUCTION

Mesenteric ischemia is a clinical entity with a mortality rate between 60% and 100% that usually requires surgical resection of the necrotic intestinal segment^[1]. Although there have been advancements in the treatment of ischemic injury, an ideal treatment has not been defined, and new options should be considered. A promising strategy is the use of anesthetic and sedative agents that might exert protective effects on the injured tissue. Ketamine is an agent that has been recommended for this purpose in clinical situations of sepsis, renal ischemia, cerebral ischemia and serious burn injuries^[2-4].

The small intestine is very sensitive to ischemic insult^[5]. Reperfusion causes additional damage through the release of free radicals, pro-inflammatory cytokines, leukotrienes and other related products^[6]. Intestinal

ischemia/reperfusion (I/R) damages the intestinal mucosa and alters gastrointestinal motility^[7]. The histological damage induced by I/R includes alterations in capillary permeability, mucosal barrier dysfunction, necrosis, epithelial shredding. The damage is reversed in a period of approximately 24 h^[8].

The NMDA (N-methyl-D-Aspartate) receptor family is a heterogeneous group of excitatory ionotropic voltage and ligand gate dependent receptors. Ketamine is a parenteral anesthetic that non-competitively blocks the NMDA receptor^[9]. It has been shown that ketamine protects various tissues from I/R injury, such as brain^[10], myocardium^[11] and skeletal muscle^[12]. To the best of our knowledge, no previous studies have evaluated the effects of ketamine on intestinal motility impairment and tissue damage induced by I/R.

MATERIALS AND METHODS

Procedures

Animal procedures were performed in accordance with the proper use and care of laboratory animals. Experiments were performed on 30 male Wistar rats weighing 200-250 g. Animals were maintained under standard conditions, such as stable room temperature ($24 \pm 3^\circ\text{C}$), a 12 h light/12 h dark cycle, and access to commercial rat pellets and water *ad libitum*.

Animal models

Briefly, after anesthesia with pentobarbital sodium (Anestesal, Pfizer Inc, Mexico) or ketamine (Anesket, Pisa Agropecuaria, Hidalgo, Mexico), midline laparotomy was performed, and the small intestine was externalized and kept wrapped in humid sterile gauze to prevent dehydration. Rats were placed over a heating mat to maintain constant core temperature (37°C). Intestinal I/R was induced by selective clamping of the vascular supply of an ileum segment consisting of 25% of the total intestinal length with microvascular clamps for 45 min (ischemia) followed by either 60 min or 24 h reperfusion. Ischemia was confirmed by absence of pulsations as well as characteristic changes in intestinal coloration.

Thirty male Wistar rats were randomized into 3 groups ($n = 10$) depending on the type of anesthetic used. Each group was then divided into 2 subgroups. In subgroup A ($n = 5$), reperfusion lasted 60 min, and tissue was obtained for histological examination only, while in subgroup B ($n = 5$) it was extended to 24 h and the animals used to evaluate intestinal transit and intestinal electrical activity.

The control group ($n = 10$) animals were anesthetized with pentobarbital sodium (50 mg/kg) and sham-operated (only midline laparotomy and gut manipulation). In the SK group ($n = 10$) animals were anesthetized with pentobarbital sodium (50 mg/kg) and intestinal I/R was induced as described. In the KET group ($n = 10$) animals were anesthetized with ketamine (100 mg/kg) and intestinal I/R was induced as described.

Intestinal transit and basic electrical rhythm

In subgroup B, after 24 h of reperfusion, animals were anesthetized with pentobarbital sodium (50 mg/kg), mid line laparotomy was performed, and 3 subserosal bipolar electrodes were used to record the electrical activity of the ileum segment that had been subjected to I/R injury using a Power Lab 4/20T (ADInstruments, USA) equipped with 3 DC pre-amplifiers (ADInstruments, USA) to analyze and calculate intestinal Basic Electrical Rhythm (BER). Immediately after 30 min, 2 mL/kg of a non-absorbable charcoal solution marker was instilled through a canula into the proximal end of the ileum segment studied, and after another 30 min the intestine was removed, and the distance traveled by the marker was registered. The distance traveled by a marker was then expressed as percentage of total intestinal length from pylorus to cecum. This was used as an indicator of intestinal transit. Finally, tissue samples were obtained for morphologic examination.

Morphological examination

After the I/R procedure, rats were sacrificed by exsanguination from the aorta. Immediately, tissue samples were obtained and fixed in 10% neutral buffered formalin and embedded in paraffin, then 5 micrometer-thick sections were stained with hematoxylin and eosin and examined under light microscope by a blinded pathologist. The Chiu scale^[13] of mucosal injury was used to evaluate the degree of histological alteration on 10 sections of 1 mm each to complete 1 cm per animal and then averaged. The scale consists of values from 0 to 5, where 0 normal mucosa; 1, development of sub epithelial (Gruenhagen's) spaces; 2, extension of the sub epithelial space with moderate epithelial lifting from the lamina propria; 3, extensive epithelial lifting with occasional denuded villi tips; 4, denuded villi with exposed lamina propria and dilated capillaries; and 5, disintegration of the lamina propria, hemorrhage, and ulceration.

Statistical analysis

Data were analyzed with SPSS 11.0 (SPSS Inc. Software, Chicago, Illinois, USA) statistical software using one-way analysis of variance (ANOVA) and with Tukey-Kramer test so as to determine comparison between groups, and differences between groups, respectively. All values were expressed as mean \pm SD and $P < 0.05$ was considered statistically significant.

RESULTS

Morphological examination

Structural changes in intestinal mucosa in rats of group KET A (ketamine anesthesia) showed moderate alteration such as epithelial lifting, while ulceration, hemorrhage and villi disintegration was observed in rats of group SK A after 45 min of ischemia and 60 min of reperfusion (Figure 1). The results of the quantitative evaluation of structural damage using the Chiu scale were as follows: control A 0.7 ± 0.45 , SK A 4.58 ± 0.50

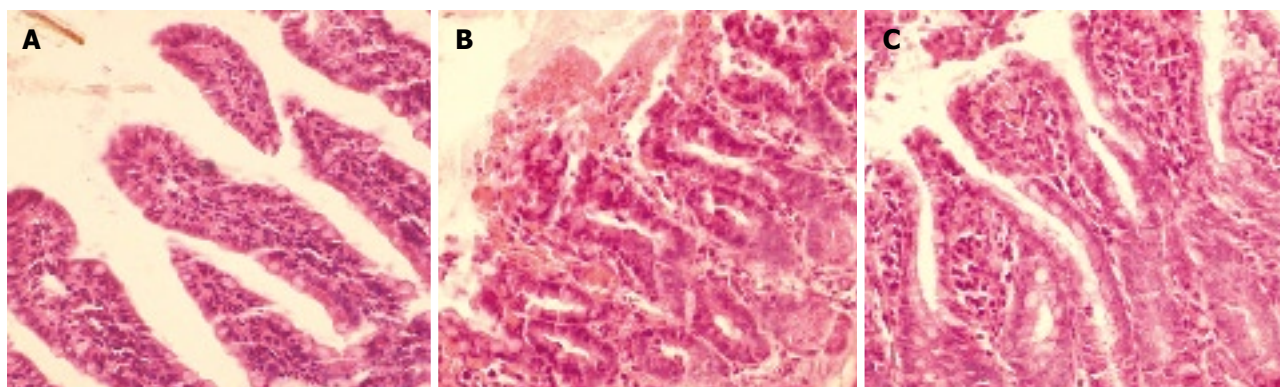


Figure 1 A: Control A group showing normal mucosa; B: SK A. Changes in intestinal mucosa structure following 45 min of ischemia and 60 min reperfusion; C: KET A. Less damage was observed (all x 40).

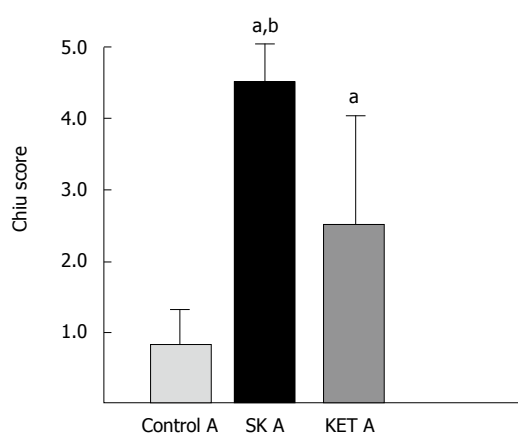


Figure 2 Bar diagram illustrating the Chiu score of mucosal injury after 60 min of reperfusion. Lower damage score was observed in a KET A group than in the SK A group. ^a $P < 0.0001$ vs control A; ^b $P < 0.0001$ vs KET A.

and KET A 2.35 ± 1.14 (Figure 2). Damage in group KET A was significantly reduced in comparison with group SK A ($P < 0.0001$). However, the damage score in the KET A group did not reach control A group levels, indicating only moderate protection. The degree of mucosal damage after 24 h of reperfusion (subgroups KET B and SK B) was the same as in control rats and showed no statistical significance.

Intestinal transit and BER

The distance traveled by the marker, expressed as percentage of total intestinal length, in subgroup SK B was $20\% \pm 2\%$ in comparison with subgroup KET B $25.9\% \pm 1.64\%$. In the control B group, the distance traveled was $27\% \pm 1.4\%$ (Figure 3). Intestinal transit showed statistically significant delay in the SK subgroup compared with the other groups in this study ($P = 0.0004$). Group KET B showed no significant alteration in transit speed compared to the control B group. The frequency of the BER was not statistically different between any of the groups after 24 h reperfusion: Control B $11.5 \pm 1.1/\text{min}$; SK B $11.2 \pm 0.5/\text{min}$; KET B $11.75 \pm 0.95/\text{min}$.

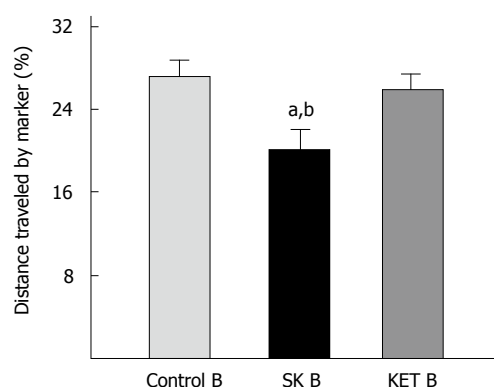


Figure 3 Bar diagram illustrating the distance travelled by the marker, expressed as a percentage of total intestinal length from pylorus to cecum. Intestinal I/R caused transit delay that was corrected by the administration of ketamine as anesthetic. ^a $P = 0.00019$ vs control B; ^b $P = 0.0004$ vs KET B.

are directly related to the length of both ischemia and reperfusion^[14]. The functional alterations caused by I/R have been previously identified^[15]. These changes consist of reversible alterations in smooth muscle contractility and intestinal transit, as well as characteristic changes in electrical activity such as a decrease in BER frequency during ischemia^[7,16]. Nitric oxide is one of the mediators thought to participate in the changes caused by I/R on intestinal motility in the rat^[17].

The structural damage caused by ischemia is aggravated by the restitution of blood flow. The physiopathology of intestinal mucosal damage by I/R is not completely understood. But, it is believed that cytotoxic substances such as free radicals, nitric oxide, serotonin, complement, as well as neutrophil infiltration and nuclear transcription factors play important roles^[18]. Macrophages have been also implicated in the initial damage caused by intestinal I/R^[19]. I/R has also been shown to induce apoptosis^[20]. Bacterial translocation and mucosal barrier dysfunction have been implicated in the damage caused by I/R in the gut^[21]. This damage is also related to an increase in circulating levels of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor alpha (TNF-alpha), liberated by the intestine itself^[22].

Recently, anti-inflammatory properties of ketamine

DISCUSSION

The alterations on intestinal motility caused by I/R

have been described in various experimental models. Interleukin-1, 6, 8 and TNF- α levels are reduced after the administration of ketamine^[23-25]. Neutrophil adhesion, migration, and free radical release is inhibited by ketamine^[26,27], as is nitric oxide production by macrophages^[28]. Further studies are needed to determine whether one of these effects is responsible for the mucosal protection against I/R that we observed in this study.

Glutamate is an excitatory neurotransmitter in the central nervous system, and it participates in the modulation of intestinal function through the enteric nervous system, where NMDA receptors have been found^[29]. It has been shown that the glutamate system is involved in the pathogenesis of I/R in brain and intestine^[30]. Glutamate release and NMDA receptor activation induces nitric oxide, and other free radicals that cause tissue injury^[31]. Glutamate release during I/R could also cause toxicity in the enteric nervous system, which could cause motility alterations^[32]. One recent study found that intestinal I/R causes myenteric plexus ganglion cell reduction and death^[33]. This suggests that the NMDA receptor could be related to the functional alterations induced in the intestine by I/R. In one study, the NMDA antagonist MK-801 diminished nitric oxide production, and prevented the changes in the intestinal transit caused by intestinal ischemia/reperfusion^[34]. We showed that ketamine, an NMDA receptor antagonist, also corrects the intestinal transit delay caused by intestinal I/R, but we did not find any changes in the frequency of the BER after 24 h of reperfusion. Further studies are needed in order to determine whether our results are explained by ketamine acting as an NMDA receptor antagonist in the gut.

Ketamine protects the intestine against other types of non-ischemic insults. Ketamine has been shown to protect the gut against endotoxemia induced injury in rats through diminished pro-inflammatory cytokine release^[35]. Ketamine also decreased enterocyte apoptosis in a model of thermal burn induced intestinal injury^[36]. However, this is the first study to demonstrate that ketamine anesthesia reduces I/R induced injury in intestinal mucosa.

COMMENTS

Background

In intestinal diseases associated with ischemia and reperfusion, intestinal tissue is damaged and intestinal function is altered. In this study, the aim was to investigate the effects of ketamine anesthesia in rats in which intestinal ischemia/reperfusion was induced.

Research frontiers

The authors suggest that ketamine anesthesia may have beneficial effects that prevent the tissue damage and functional alterations that follow from intestinal ischemia/reperfusion.

Innovations and breakthroughs

This study is the first to show that ketamine anesthesia can reduce the intestinal tissue damage induced by ischemia reperfusion.

Applications

Ketamine should be further studied and the possibility of using ketamine as anesthetic in cases of intestinal ischemia should be considered.

Peer review

Methods and results are clear, but in order for the authors to come to the

conclusion that ketamine is functioning as an NMDA receptor antagonist the following experiment needs to be done: Compare the effect of pentobarbital plus a selective NMDA antagonist vs. ketamine plus a selective NMDA antagonist. If the authors' conclusion is correct the selective NMDA receptor antagonist should "nullify" the beneficial effect of ketamine.

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Maintenance time of sedative effects after an intravenous infusion of diazepam: A guide for endoscopy using diazepam

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gastrointestinal endoscopy in Japan.

METHODS: Fifteen healthy Japanese volunteers consisting of three different *CYP2C19* genotype groups underwent a critical flicker fusion test, an eye movement analysis and a postural sway test as a test for physical sedative effects, and a visual analog scale (VAS) symptom assessment method as a test for mental sedative effects during the 336 h period after the intravenous infusion of diazepam (5 mg).

RESULTS: The physical sedative effects assessed by the critical flicker test continued for 1 h (t values of 5 min, 30 min and 60 min later: 4.35, 5.00 and 3.19, respectively) and those by the moving radial area of a postural sway test continued for 3 h (t values of 5 h, 30 h, 60 min and 3 h later: -4.05, -3.42, -2.17 and -2.58, respectively), which changed significantly compared with the baseline level before infusion ($P < 0.05$). On the other hand, the mental sedative effects by the VAS method improved within 1 h. The *CYP2C19* genotype-dependent differences in the postinfusion sedative effects were not observed in any of the four psychomotor function tests.

CONCLUSION: With the psychomotor tests, the objective sedative effects of diazepam continued for 1 h to 3 h irrespective of *CYP2C19* genotype status and the subjective sedative symptoms improved within 1 h. Up to 3 h of clinical care appears to be required after the infusion of diazepam, although patients feel subjectively improved.

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Key words: Diazepam; Sedation; cytochrome P450 (CYP) 2C19; Endoscopy; Complication; Critical flicker fusion test; Eye movement analysis; Postural sway test; Visual analog scale

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Abstract

AIM: To examine whether the sedative effects assessed by psychomotor tests would depend on the cytochrome P450 (*CYP*) 2C19 genotypes after an infusion regimen of diazepam commonly used for

INTRODUCTION

Gastrointestinal endoscopy is commonly performed for the screening and/or diagnosis of upper gastrointestinal disorders. Recent advances in gastrointestinal endoscopy are remarkable, and gastrointestinal endoscopy now plays an important role in the treatment of several upper gastrointestinal disorders (e.g. endoscopic mucosal resection of gastric cancer in the early stage and treatment of haemorrhage from peptic ulcer). However, gastrointestinal endoscopy is invasive and accompanied with distress of patients. Therefore, benzodiazepines, such as diazepam and midazolam, are commonly used as a premedication for gastrointestinal endoscopy^[1,2]. The administration of benzodiazepines as premedications for endoscopy reduces distress of patients and results in a better patient acceptance and compliance for endoscopy^[1,2]. However, use of benzodiazepines sometimes causes undesirable events; e.g. residual benzodiazepine after endoscopy may cause sustained distraction, which leads to some accidents. For the prevention of such undesirable accidents, the dose of benzodiazepines should, therefore, be minimized or optimized. However, a reduced dose of benzodiazepines sometimes results in an insufficient sedation for endoscopy, which is a dilemma for performing the safe and comfortable endoscopy.

Diazepam is metabolized by cytochrome P450 (CYP) 3A4 to temazepam, and by CYP3A4 and CYP2C19 to N-desmethyldiazepam^[3,4]. There are genetic differences in the activity of CYP2C19^[3,4]. The pharmacokinetics of diazepam significantly depend on *CYP2C19* genotype status^[5-9], as observed in proton pump inhibitors, such as omeprazole and rabeprazole^[10,11]. The *CYP2C19* genotypes are classified into the three groups: homozygous extensive metabolizers (EMs), heterozygous EMs, and poor metabolizers (PMs)^[12-14]. In PMs, the plasma diazepam concentrations are markedly increased due to an impaired metabolism of diazepam in comparison with those in EMs^[3-7]. However, whether the pharmacodynamic effects of an intravenous infusion of diazepam would differ between the *CYP2C19* EMs and PMs remain, to our knowledge, unknown.

Psychomotor tests, such as a critical flicker fusion test, an eye movement analysis, a postural sway test and a visual analog scale (VAS) symptom assessment method are well known as psychometric markers and are commonly used to quantify the pharmacodynamic responses associated with an administration of benzodiazepine sedatives^[15-17]. Indeed, plasma or serum diazepam concentrations were significantly correlated with psychomotor test scores^[18,19].

In this study, we aimed to examine the objective and subjective sedative effects of an intravenous 5-mg dose of diazepam commonly used for gastrointestinal endoscopy in relation to *CYP2C19* genotypes.

genotypes had been determined by a PCR-RFLP method^[20,21] were enrolled in this study (5 homozygous EMs, 5 heterozygous EMs and 5 PMs of *CYP2C19*) (Table 1). A written informed consent was obtained from each subject.

Study protocol

All subjects were given a single intravenous infusion of Diazepam (Cercine®, Takeda Pharmaceutical Co. Ltd., Osaka, Japan) 5 mg at 8:00 am, which was infused over 1 min. Four psychomotor tests, such as a critical flicker fusion test, an eye movement analysis, a postural sway test and a VAS symptom assessment method^[15-17], were performed at the pre- and postinfusion time points of diazepam as follows: before the infusion and 5 min and 30 min, and 1 h, 3 h, 6 h, 10 h, 24 h, 72 h and 336 h postinfusion.

The critical flicker fusion test was measured by the discrimination of fused flickering red light (DF-1, Shibata Chemical, Tokyo, Japan). The value used for the fusion time was flickers per second. The results of an eye movement test used the saccadic latency (per second), which is time from the displacement of red light signal to the response of eye movement. The stimulated horizontal displacement of red lights at random intervals was recorded (DP1200A, Nihon Denki Sanei, Tokyo, Japan). The subjects responded to and followed a signal for 30 s as quickly as possible. The moving radial area (cm²) for 60 s by a postural sway test was measured by a sway meter (G5500, Anima, Tokyo, Japan) for the 60 s period with eyes closed. The radial area was determined by calculating the radial distance to the center of pressure at each sampling interval from the geometric center of the stance. A VAS symptom assessment method consisted of questions of both the two parameters of mental sedation and physical sedation. Subjects had to mark on the 100 mm line to show the degree of their feeling.

All subjects were provided with three meals a day (breakfast at 7:30 am, lunch at 12:30 pm, and supper at 6:00 pm). Mineral water was allowed *ad libitum*. But, no other beverages including caffeine-containing and grapefruit juice-related products were permitted. None of them drank alcohol or had a smoking habit. None had taken any drugs for 1 mo before the study, nor did they take any during it. The subjects stayed in the translational research (TR) unit of Hamamatsu University School of Medicine during the first 24 h postinfusion period. Four psychomotor tests at 72 h and 336 h postinfusion were performed in the TR unit.

The protocol was approved in advance by the Human Institutional Review Board of the Hamamatsu University School of Medicine. Written informed consent was obtained from each subject before participation in the study.

Statistical analysis

The median values of four psychomotor tests and median percent changes from the baseline value (before Diazepam infusion) were determined. Statistically

MATERIALS AND METHODS

Subjects and *CYP2C19* genotyping

Fifteen healthy Japanese volunteers whose *CYP2C19*

Table 1 Demographic characteristics of healthy volunteers with different *CYP2C19* genotype status ($n = 5$)

	Homozygous EM	Heterozygous EM	PM	P
Genotype status	Wild-type/Wild-type ($n = 5$)	Wild-type/ <i>CYP2C19</i> mutation in exon 5 ($n = 3$) Wild-type/ <i>CYP2C19</i> mutation in exon 4 ($n = 2$)	<i>CYP2C19</i> mutation in exon 5/ <i>CYP2C19</i> mutation in exon 5 ($n = 2$) <i>CYP2C19</i> mutation in exon 4/ <i>CYP2C19</i> mutation in exon 4 ($n = 1$) <i>CYP2C19</i> mutation in exon 5/ <i>CYP2C19</i> mutation in exon 4 ($n = 2$)	
Male/Female	3/2	3/2	5/0	0.472
Age (yr)	24 (22-31)	29 (25-44)	23 (22-35)	0.093
Height (cm)	170 (154-174)	177 (155-181)	171 (169-180)	0.400
Body weight (kg)	62 (51-67)	67 (51-78)	65 (57-78)	0.424

Age and body weight are given as median (range). EM: Extensive metabolizer; PM: Poor metabolizer.

significant differences in the median pharmacodynamic parameters among the 3 different *CYP2C19* genotype groups at the pre- and postinfusion time points were determined by the Mann-Whitney *U*-test, when a significant difference was obtained by the Kruskal-Wallis test. Statistical differences with the median parameters between the different pre- and postinfusion time points in all of the subjects enrolled in this study were determined by using the Wilcoxon's signed rank test, when significant differences were obtained by the Friedmann's test. All *P* values were two-sided, and $P < 0.05$ was taken to indicate statistical significance.

RESULTS

There were no statistically significant differences in the demographic characteristics, such as age, body weight, height and sex ratio among the 3 different *CYP2C19* genotype groups (Table 1).

Objective sedative effects assessed by critical flicker fusion, postural sway and eye movement tests

Percent changes in the critical flicker fusion test, postural sway test, and eye movement test after an intravenous administration of 5 mg of diazepam as a function of *CYP2C19* genotype status are shown in Figure 1. Sedative effects with diazepam (5 mg) assessed by those methods did not significantly differ among the different *CYP2C19* genotype groups throughout the 336 h postinfusion period ($P > 0.05$) (Figure 1). For this reasoning, the data derived from the different *CYP2C19* genotypes were combined together in the following analyses.

In a critical flicker fusion test, the median percent changes from the baseline values (before diazepam infusion) and the postinfusion values at 5 and 30 min, and at 1 h significantly differed ($P = 0.0038$, 0.0018 and 0.0090 , respectively) (Figure 2A). The median percent changes from the baseline values still appeared increased for longer than 3 h postinfusion, but not significantly (Figure 1A).

In the moving radial area of a postural sway test, the median values at 5 and 30 min, and 1 h and 3 h postinfusion were 167.9% (range; 100.3%-456.7%), and 153.2% (52.3%-391.0%), and 111.2% (75.6%-281.8%) and 130.8% (51.0%-337.1%), which were significantly greater than the baseline value ($P = 0.0007$, and 0.0018 ,

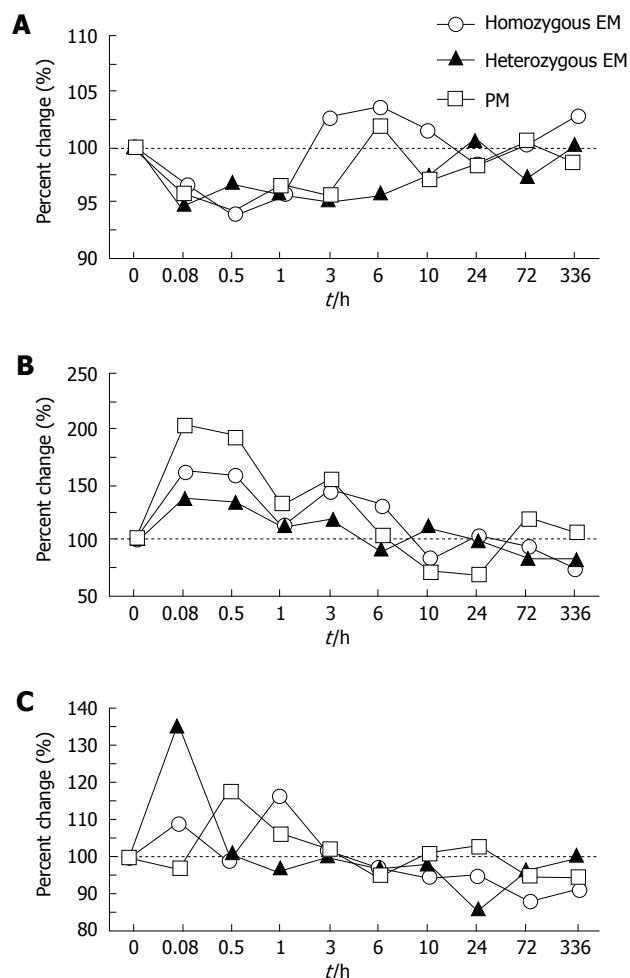


Figure 1 Median percent changes from the baseline value (before a diazepam 5 mg infusion) by a critical flicker fusion test (A), a postural sway test (B), and an eye movement test (C) in the 3 different *CYP2C19* genotype groups.

and 0.0409 and 0.0125 , respectively) (Figure 2B). These objective sedative assessment values required 3 h to 6 h postinfusion to return to the respective baseline levels (Figure 2).

In the saccadic latency of an eye moving test, the median percent change from the baseline at 5 min postinfusion was 115.9% (81.9%-141.1%) ($P = 0.0171$) (Figure 2C).

The overall results indicated that the objective

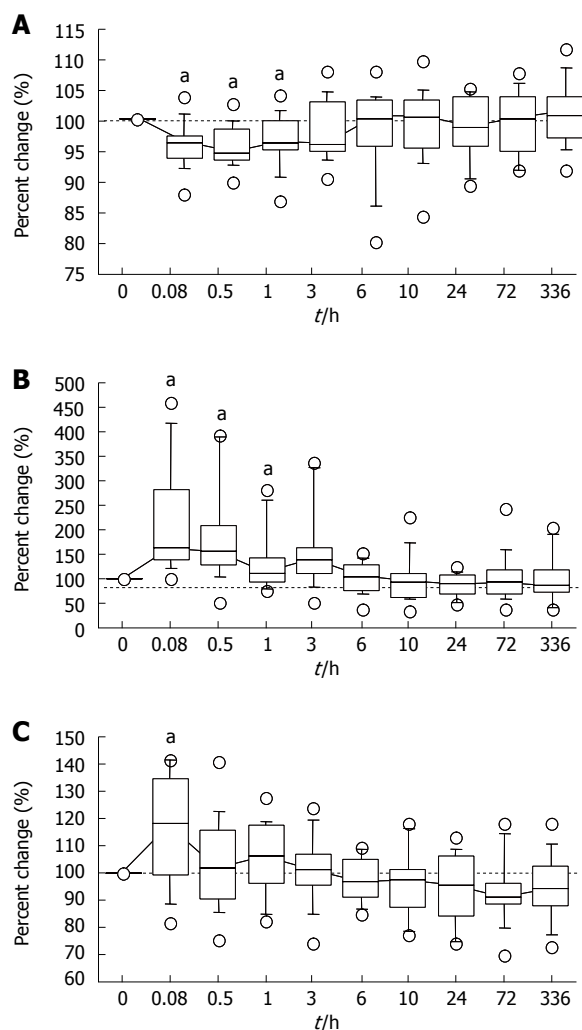


Figure 2 Median percent changes from the baseline value [before a diazepam (5-mg) infusion] by a critical flicker fusion test (A), a postural sway test (B), and an eye movement test (C) in all of the enrolled subjects. ^a $P < 0.05$ by using the Wilcoxon's signed rank test, when significant differences were obtained by the Friedmann's test.

sedative effects of diazepam assessed by the three methods continued for up to 3 h postinfusion.

Subjective sedative effects assessed by the VAS method

The sedative effects assessed by VAS method were fairly similar among the different *CYP2C19* genotype groups throughout the 336-h postinfusion period ($P > 0.05$) (Figure 3). The data of VAS method obtained from all of the subjects with different *CYP2C19* genotypes were, therefore, combined as mentioned above.

The median values by “alert to drowsy” of mental sedation parameter at 5 and 30 min postinfusion were significantly decreased compared with the baseline value ($P = 0.0098$ and 0.0047 , respectively) (Figure 4A). This value returned to the level not different from the baseline of 1 h later (Figure 4A). In the “fuzzy to clear headed” of mental parameter, the median value at 5 min later was significantly lower ($P = 0.076$), but returned to the baseline at 30 min postinfusion (Figure 4B).

The median values of the “well-coordinated to clumsy” physical sedation parameter at 5 min and 30 min and 1 h after diazepam infusion were significantly

increased compared with the baseline value ($P = 0.0031$, and 0.0038 and 0.0468 , respectively) (Figure 4C). In the “lethargic to energetic” physical parameter, the median values at 5 min and 30 min postinfusion were significantly lower ($P = 0.0119$ and 0.0468 , respectively), but returned to baseline level by 1 h postinfusion (Figure 4D).

These results assessed by the VAS symptom assessment method indicated that the subjective sedative effects of diazepam (5 mg) continued for less than 1 h postinfusion, although the objective sedative effects continued longer (for 1 h to 3 h postinfusion).

DISCUSSION

The development of an optimal infusion regimen of a premedication (e.g. diazepam) and an appropriate manual check at the endoscopy unit is necessary for a comfortable and safe gastrointestinal endoscopy. Although the proper premedication for gastrointestinal endoscopy should have a shorter onset of action, short elimination half-life, and faster time to recovery, diazepam is one of the first-line sedative drugs used as a premedication for gastrointestinal endoscopy in Japan. In this study, we demonstrated that the objective sedative effects by an intravenous infusion of diazepam (5 mg) continued for no less than 3 h with psychological tests, whereas the subjective sedative effects continued for no more than 1 h. Although the plasma diazepam concentrations of individuals with *CYP2C19* PMs have been reported to be greater than those with homozygous EMs^[5-9], we did not find any significantly different sedative effects of diazepam among the different *CYP2C19* genotype groups. Based upon these observations, we thought that patients who undergo an intravenous infusion of diazepam (5 mg) should be cared for at least 3 h postinfusion in the hospital irrespective of *CYP2C19* genotype status for the prevention of adverse events.

In the present study, the values of VAS method used for assessing the subjective symptom parameters demonstrated continuous sedative effects only during 30 min to 60 min after the infusion of diazepam (5 mg). After 1 h had passed from administration, the major parameters of the VAS assessment method returned to the respective baseline levels. However, the objective assessment by a critical flicker fusion test and a postural sway test revealed that the sedative effects of diazepam remained for approximately 3 h to 6 h postinfusion, indicating that a discrepancy exists between the subjective and objective sedative symptoms from 1 h to 6 h after the infusion of diazepam. Therefore, even if the patients feel improved or recovered from the sedation by diazepam at 1 h postinfusion, they may be at risk for some adverse effects, such as falling down and/or having driving errors. Then, we anticipate that the patients who undergo an intravenous infusion of diazepam (5 mg) should be checked for the prevention of possible sedative adverse effects during a 6 h postinfusion period at the endoscopy unit.

The sedative effects of diazepam are mediated via $\alpha 1$ -GABA_A receptors in the brain^[22,23], and the minimum plasma concentration of diazepam to yield sedative

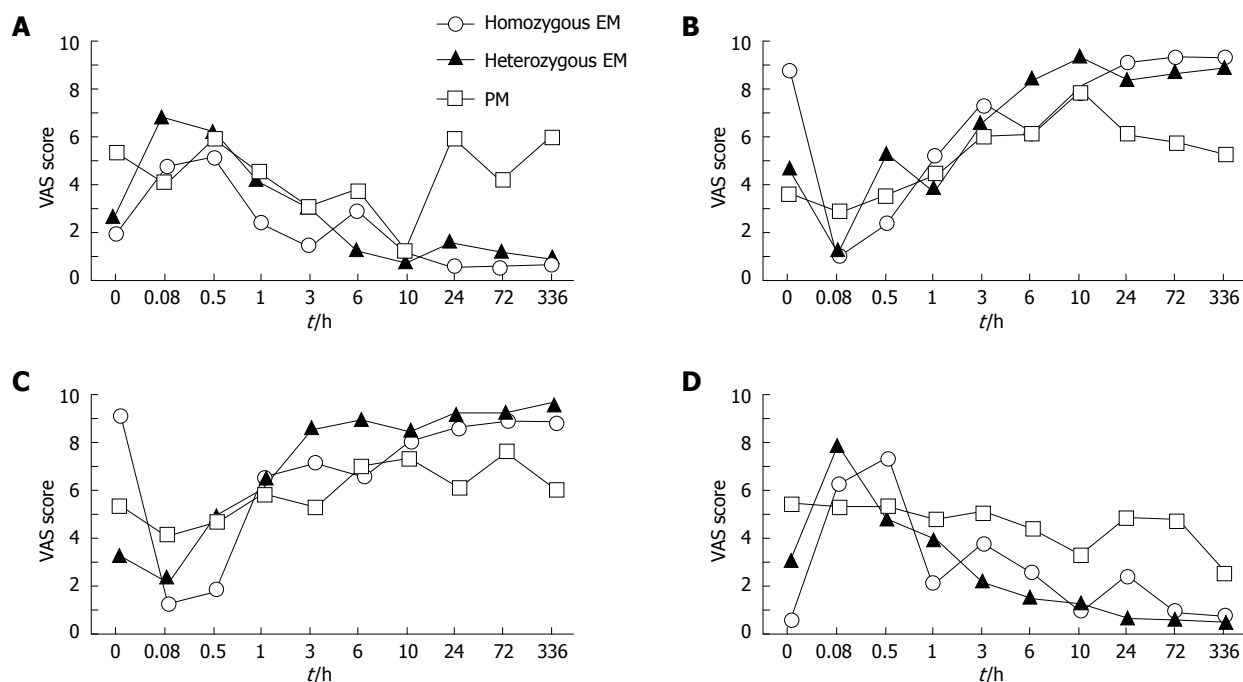


Figure 3 Median percent values for mental sedation, "alert to drowsy" (A), "fuzzy to clear-headed" (B), physical sedation, "lethargic to energetic" (C) and "well-coordinated to clumsy" (D) parameters by a VAS symptom assessment test in the 3 different *CYP2C19* genotype groups.

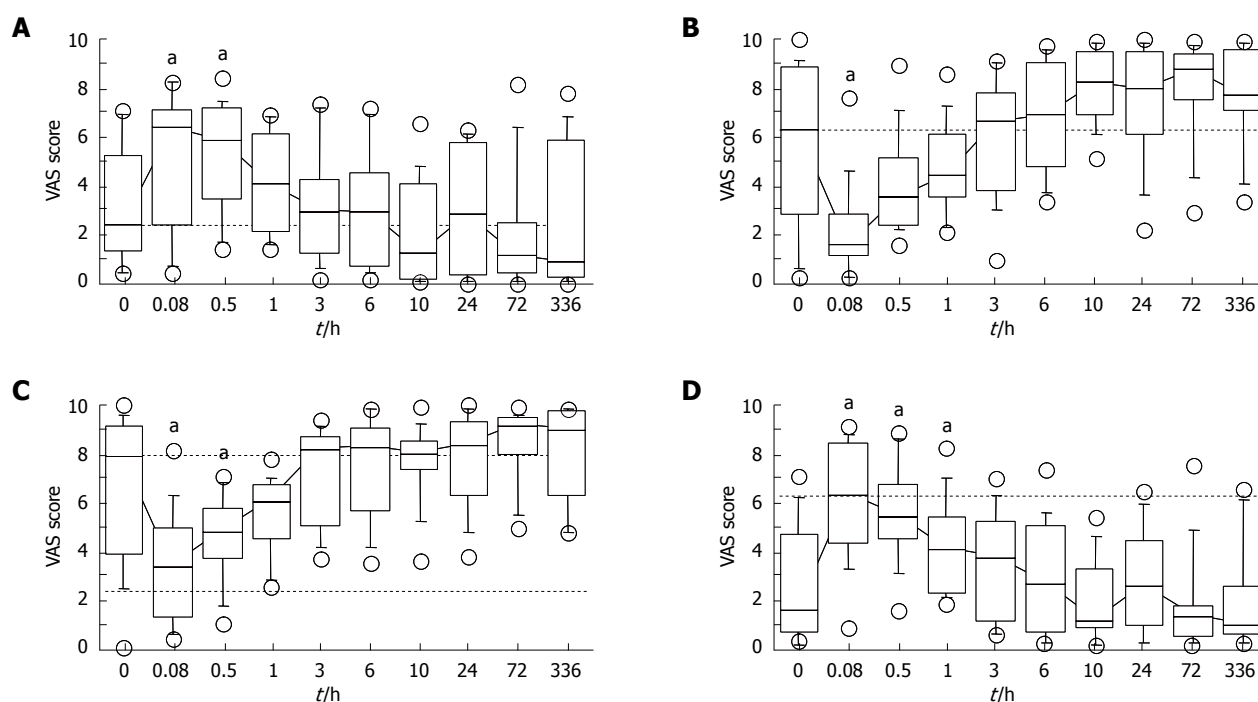


Figure 4 Median percent values for mental sedation "alert to drowsy" (A), "muzzy to clear-headed" (B), physical sedation "lethargic to energetic" (C) and "well-coordinated to clumsy" (D) parameters by a VAS symptom assessment test in all of the enrolled subjects. ^a*P* < 0.05 by using the Wilcoxon's signed rank test, when significant differences were obtained by the Friedmann's test.

effects is more than 300 ng/mL to 400 ng/mL^[18,19]. The pharmacokinetics of diazepam are affected by age^[24,25], gender^[26], obesity^[26], liver disease^[27] and *CYP2C19* genotype status^[5-9]. The mean plasma elimination half-life of diazepam in PMs of *CYP2C19* has been reported to be much longer than that in homozygous EMs: those values after a single oral dose of diazepam were 84.0 ± 13.7 h for PMs, 62.9 ± 9.8 h for heterozygous EMs

and 20.0 ± 10.8 h for homozygous EMs^[28]. However, plasma diazepam levels do not always correlate with the sedative effects^[29,30]. Moreover, Kosuge *et al.*^[16] reported that influences of diazepam on psychomotor functions did not differ among the 3 different *CYP2C19* genotype groups, as observed in this study. Although we cannot offer appropriate explanations for the lack of difference in the pharmacodynamics of diazepam among the different

CYP2C19 genotype groups, several possible mechanisms can be raised as follows: first, the acute tolerance to diazepam may be developed, which is mediated by the dysfunction of the cortical GABA transmitter system, such as the decrease of glutaminic acid decarboxylase^[31,32], reelin^[33] and GABA membrane transporter^[34], GABAA receptor up-regulation^[35] and the decrease of dendritic spines^[36]. Second, diazepam as well as its metabolites such as temazepam and N-desmethyldiazepam, have sedative effects^[3,4]. Therefore, although the metabolic disposition of diazepam differs among the different *CYP2C19* genotype groups, the total amounts of diazepam plus its active metabolites would not differ among the different *CYP2C19* genotype groups, resulting in no statistical difference in the pharmacodynamics of diazepam. Third, our pharmacodynamic assessment methods may not have a sufficient power for the limited sample size ($n = 15$) in the psychomotor function status. Nevertheless, in a cimetidine-diazepam interaction study, only minimal changes were observed in the pharmacodynamic effects despite an increase in plasma diazepam concentration by about 60% during treatment with cimetidine^[37]. Similarly, in a fluoxetine-diazepam interaction study^[38], despite that the significant increment of plasma diazepam concentration occurred by about 50% with the co-administration of fluoxetine, no psychopharmacological changes were detected by the pharmacodynamic assessment methods similar to those we used. With the limitation of absent data on plasma concentrations of diazepam and its metabolites (e.g. desmethyldiazepam and temazepam) in this study, we are tempted to assume that the sedative effects assessed by the pharmacodynamic assessment methods we used would not differ among the different *CYP2C19* genotype groups when given an intravenous 5-mg infusion of diazepam as noted above.

Recently, when gastrointestinal endoscopy is performed for patients, midazolam and propofol are often used as a sedative drug. However, midazolam and propofol act quickly and potently, and therefore, advanced effects such as respiratory trouble occurs sometimes. However, the proper premedication for gastrointestinal endoscopy should have a shorter onset of action, short elimination half-life, and faster time to recovery. Therefore, we hope to evaluate the sedative effects of those drugs by using the psychopharmacological tests.

In conclusion, this study suggests that the psychopharmacological tests appear to be a useful tool for determining the optimal treatment with a benzodiazepine, such as diazepam, for gastrointestinal endoscopy. If patients undergo gastrointestinal endoscopy for sedation by an intravenous infusion of diazepam (5 mg), the sedative effects of patients should be monitored for preventing the possible adverse effects up to at least a 3-h postinfusion period in the endoscopy unit of the hospital, although patients appear to subjectively feel unimpaired or recovered.

COMMENTS

Background

Benzodiazepine is commonly used as a premedication for gastrointestinal

endoscopy. However, use of benzodiazepine sometimes causes undesirable events, which leads to some accidents. For the prevention of such undesirable accidents, therefore, the dose of premedication should be minimized or optimized. For benzodiazepine, the pharmacokinetics of diazepam depends significantly on cytochrome P450 (*CYP*) 2C19 genotype status. However, whether the pharmacodynamic effects of an intravenous infusion would differ between the *CYP2C19* EMs and PMs remain unknown. Moreover, there was no optimal protocol of diazepam use in the endoscopy unit.

Research frontiers

When patients receive gastrointestinal endoscopy, the development of optimal infusion regimens of sedative drug and care protocol in the endoscopy unit are required to prevent advanced effects in relation to *CYP2C19* genotypes.

Innovations and breakthroughs

There are many reports, which investigate the pharmacokinetics and pharmacodynamics of diazepam in pharmacological studies. The innovation of this study is to demonstrate the recommendation and attention of gastrointestinal endoscopy with diazepam (5 mg/body) as follows: An up to 3 h clinical care appears to be required after the infusion of diazepam irrespective of *CYP2C19* genotype status, although patients feel subjectively improved.

Applications

The physical sedative effects assessed by the critical flicker test continued for 1 h and those by the moving radial area of a postural sway test continued for 3 h, which significantly changed compared with the baseline level before infusion ($P < 0.05$). On the other hand, the mental sedative effects by the VAS method improved within 1 h. The *CYP2C19* genotype-dependent differences in the postinfusion sedative effects were not observed in any of the four psychomotor function tests. Therefore, up to 3-h of clinical care is required after the infusion of diazepam (5 mg) irrespective of *CYP2C19* genotype status, although patients feel markedly improved. Recently, many drugs, such as midazolam and propofol, were used at endoscopy as sedation drugs. Additional research, which compares with diazepam and other sedation drugs in efficacy and care time using the psychopharmacological tests, is required.

Terminology

The critical flicker fusion test: the measurement of the discrimination of fused flickering red light. The value used for the fusion time was flickers per second. The results of an eye movement test: the saccadic latency (per second), which is time from the displacement of red light signal to the response of eye movement. The subjects responded to and followed a signal for 30 s as quickly as possible. The postural sway test: the measurement of moving radial area (cm^2) for the 60 s period with eyes closed. The radial area was determined by calculating the radial distance to the center of pressure at each sampling interval from the geometric center of the stance.

Peer review

Authors examined whether the sedative effects assessed by psychomotor tests would depend on the *CYP2C19* genotypes after an infusion regimen of diazepam commonly used for gastrointestinal endoscopy in Japan. It's an interesting study, and the methodology is sound, and is a good objective assessment.

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RAPID COMMUNICATION

***Nigella sativa* relieves the deleterious effects of ischemia reperfusion injury on liver**

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Abstract

AIM: To determine whether *Nigella sativa* prevents hepatic ischemia-reperfusion injury to the liver.

METHODS: Thirty rats were divided into three groups as sham (Group 1), control (Group 2), and *Nigella sativa* (NS) treatment group (Group 3). All rats underwent hepatic ischemia for 45 min followed by 60 min period of reperfusion. Rats were intraperitoneally infused with only 0.9% saline solution in group 2. Rats in group 3 received NS (0.2 mL/kg) intraperitoneally, before ischemia and before reperfusion. Blood samples and liver tissues were harvested from the rats, and then the rats were sacrificed. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were determined. Total antioxidant capacity (TAC), catalase (CAT), total oxidative status (TOS), oxidative stress index (OSI) and myeloperoxidase (MPO) in hepatic tissue were measured. Also liver tissue histopathology was evaluated by light microscopy.

RESULTS: The levels of liver enzymes in group 3 were

significantly lower than those in the group 2. TAC in liver tissue was significantly higher in group 3 than in group 2. TOS, OSI and MPO in hepatic tissue were significantly lower in group 3 than the group 2. Histological tissue damage was milder in the NS treatment group than that in the control group.

CONCLUSION: Our results suggest that *Nigella sativa* treatment protects the rat liver against to hepatic ischemia-reperfusion injury.

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Key words: *Nigella sativa*; Ischemia reperfusion injury; Liver

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INTRODUCTION

Ischemia followed by reperfusion (I/R) may cause metabolic and structural hepatic damage, and may be due to trauma, sepsis, liver transplantation^[1] or hepatic pedicle clamping during liver surgery^[2]. This remains a significant problem for surgical procedures, and also remains limitation of liver transplantation^[3].

Oxygen free radicals, produced on reperfusion, play a critical role in the injury caused by ischemia-reperfusion^[4]. Reactive oxygen radicals lead to an inflammatory response and tissue damage by activating some mediators. It can also directly damage cell components^[5]. Several attempts to reduce these mechanisms have been reported in the literature. Protection against reperfusion injury can be induced by assorted treatments including administration of antioxidants and anti-inflammatory drugs^[4,6-8].

Various therapeutic effects, such as antioxidant, anti-inflammatory, anticancer^[9], antihistaminic^[10], antibacterial effects^[11] have been described for *Nigella sativa*. Additionally, it has been shown that *Nigella sativa* has protective effect against ischemia reperfusion injury to various organs^[12-14]. Thymoquinone, the active constituent of *Nigella sativa* seeds, is a pharmacologically active quinone, which possesses several properties including analgesic and anti-inflammatory actions^[15]. It has been reported that thymoquinone prevents oxidative injury in various *in vitro* and *in vivo* studies in rats^[16,17]. It has been suggested that thymoquinone may act as an antioxidant agent and prevents membrane lipid peroxidation in tissues^[18]. The mechanism of action is still largely unknown. But, it seems these effects may be related to inhibition of eicosanoid generation, namely thromboxane B2 and leucotrienes B4 (by inhibiting cyclooxygenase and 5-lipoxygenase, respectively), and membrane lipid peroxidation^[13].

Moreover, it has been demonstrated that *Nigella sativa* can significantly prevent hepatotoxicity^[19] and might have protective effects against nephrotoxicity induced by either disease or chemicals^[13]. But, the exact mechanism is not clear. There are also several clinical studies. In one study, the prophylactic effect of boiled extract of *N. sativa* on asthmatic disease was examined^[20]. Similarly, black seed oil was shown to be an effective adjuvant for the treatment of patients with allergic diseases^[21]. In another clinical study, significant benefits of *Nigella sativa* extract in the treatment of acute tonsillopharyngitis was shown^[22]. Also, it was shown that *Nigella sativa* has anti-epileptic effects in children with refractory seizures^[23].

Therefore, it seems possible that the administration of *Nigella sativa* might protect the liver against the ischemia reperfusion injury; therefore, our aim was to confirm this hypothesis. We investigated alterations in the oxidant- antioxidant balance by measuring oxidant parameters such as total oxidative status (TOS), oxidative stress index (OSI) and myeloperoxidase (MPO), and antioxidant parameters, such as total antioxidant capacity (TAC) and catalase (CAT) in the liver tissue. Also we examined histopathological changes in the liver parenchyma.

MATERIALS AND METHODS

Thirty male Wistar-albino rats weighting 190 g to 250 g were used in this experimental study. All animals were maintained under standard conditions, and were fed water and rodent chow *ad libitum* and treated in compliance with the National Institutes of Health guidelines. Rats were deprived of food, but not water, for 24 h before surgery.

Animals were divided into three groups, sham group (Group 1), control group (Group 2), and *Nigella sativa* treatment group (Group 3). All rats were anesthetized with 0.2 mL/100 g of ketamine hydrochloride intraperitoneally. After the abdomen was shaved and disinfected, a midline incision was made and rats underwent either sham surgery or ischemia-reperfusion. Ischemia was carried out by exposing the afferent and efferent blood ves-

sels and then clamping for 45 min with a microvascular “bulldog” clamp. Forty five minutes later, the ischemic liver was reperfused by opening the clamp, and reperfusion was achieved for 60 min. *Nigella sativa* was given to the rats in treatment group, before ischemia and before reperfusion at a dose of 0.2 mL/kg by intraperitoneal route. We chose the dose of this agent according to reported studies about I/R and *Nigella sativa*, as this dose has been shown to be effective in previous studies^[24,25]. Rats in the control group were infused only with saline. At the end of the procedures, the rats were killed and blood and liver tissue samples were obtained. A portion of liver was stored at -80°C for future analyses. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities were measured for evaluating the liver functions. To assess oxidative injury, TAC, TOS and OSI levels were determined.

OSI and MPO levels

The enzyme analyses of liver tissue were performed on the supernatant fractions of the tissue homogenates. The tissues were homogenized in 150 mmol/L ice-cold KCl to make a 10% homogenate, using a glass Teflon homogenizer. Then, the samples were sonicated on ice ten times for 5 s. The homogenates were centrifuged at 12500 g for 30 min at 3°C, and the supernatant fractions were obtained.

Tissue samples for histological staining were obtained and fixed in 10% formalin-phosphate-buffered saline at 4°C overnight. The samples were dehydrated and embedded in paraffin. Sections (7 µm) were cut and stained with hematoxylin and eosin. A pathologist evaluated the slides in a blinded manner.

Biochemical analyses

Plasma was used to measure AST, ALT and LDH as indicative parameters of hepatic function. The plasma activities of AST, ALT and LDH were estimated by commercially available kits using an autoanalyser (aeroset® Abbott Laboratories, Chicago, IL).

Measurement of the total antioxidant capacity

TAC of supernatant fractions was determined using a novel automated measurement method developed by Erel^[26]. In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequential produced radicals such as brown colored dianisidiny radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, antioxidative effect of the sample against the potent-free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has excellent precision values, lower than 3%. The results are expressed as nmol Trolox Equiv./mg protein.

Measurement of total oxidant status

TOS of supernatant fractions was determined using a

novel automated measurement method, developed by Erel^[27]. Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of nmol H₂O₂ Equiv./mg protein.

Oxidative stress index

Percent ratio of TOS level to TAC level was accepted as OSI. OSI value was calculated according to the following formula^[28]: OSI (Arbitrary Unit) = TOS (nmol H₂O₂ Equiv./mg protein)/TAC (nmol Trolox Equiv./mg protein).

Determination of myeloperoxidase activity

The MPO (EC 1.11.1.7) activity was determined, using a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H₂O₂ and changes in absorbance at 510 nm were recorded^[29]. One unit of MPO activity was defined as that which degraded 1 mol H₂O₂/min at 25°C. The results were expressed as mU/g protein.

Determination of catalase activity

Liver catalase activity was determined by Goth's colorimetric method, in which supernatant was incubated in H₂O₂ substrate, and the enzymatic reaction stopped by the addition of ammonium molybdate. The intensity of the yellow complex formed by molybdate and H₂O₂ was measured at 405 nm^[30].

Histopathologic evaluation

Liver tissues were embedded in paraffin, cut into 3 to 5-μm sections, and mounted. After deparaffinization, the tissues were stained with hematoxylin and eosin (HE) for histological examination. Histological examination was performed by a pathologist.

Statistical analysis

For statistical analyses, nonparametric independent group comparisons were made. For multiple comparisons, the Kruskal-Wallis was used for comparisons between groups and the Mann-Whitney test used if any statistical significance was found. A level of 5% ($P < 0.05$) was considered statistically significant. Data were expressed as median and range.

RESULTS

Plasma ALT, AST, and LDH levels in the *Nigella sativa* treatment group were significantly lower than those in the control and sham groups ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively, and $P < 0.01$ for all). They were significantly higher in the control group than those in

Table 1 Clinical parameters, oxidative and antioxidative parameters in sham, I/R and I/R + NS rats ($n = 10$, mean \pm SD)

	Sham	I/R	I/R + NS	P
Clinical parameters				
AST (U/L)	132 \pm 22	952 \pm 251 ^b	571 \pm 137 ^{d,f}	0.001
ALT (U/L)	86 \pm 17	695 \pm 206 ^b	321 \pm 128 ^{d,f}	0.001
LDH (U/L)	534 \pm 181	4334 \pm 760 ^b	3113 \pm 729 ^{d,e}	0.001
TAC (nmol Trolox Equiv./mg protein)	2.96 \pm 0.4	2.17 \pm 0.6 ^a	3.07 \pm 0.2 ^{e,1}	0.029
TOS (nmol H ₂ O ₂ Equiv./mg protein)	10.4 \pm 2.2	15.9 \pm 2.0 ^b	12.8 \pm 3.1 ^{1,2}	0.003
OSI (Arbitrary Unite)	3.54 \pm 0.7	7.76 \pm 1.9 ^b	3.76 \pm 0.6 ^{1,2}	0.002
MPO (U/g protein)	9.4 \pm 1.8	13.2 \pm 1.7 ^b	11.2 \pm 2.2 ^{1,1}	0.004
CAT (U/mg protein)	18.4 \pm 3.9	10.1 \pm 1.9 ^b	18.3 \pm 2.2 ^{1,2}	0.004

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; TAC: Total antioxidant capacity; TOS: Total oxidative status; OSI: Oxidative stress index; MPO: Myeloperoxidase; CAT: Catalase. ^a $P < 0.05$, ^b $P < 0.01$, Sham groups vs I/R groups; ^d $P < 0.01$, Sham groups vs I/R + NS groups; ^e $P < 0.05$, ^f $P < 0.01$, I/R groups vs I/R + NS groups. ¹ $P > 0.05$, Sham groups vs I/R + NS groups; ² $P < 0.001$, I/R groups vs I/R + NS groups.

the sham group ($P < 0.01$ for all). The results are summarized in Table 1.

TAC and CAT activities in liver tissue were significantly higher in Group 3 than those in Group 2 ($P < 0.05$ and $P < 0.001$, respectively). However, TAC and CAT activities in liver tissue were significantly lower in Group 2 than those in Group 1 ($P < 0.05$ and $P < 0.01$, respectively). TOS and OSI in hepatic tissue were significantly lower in Group 3 than those in Group 2 ($P < 0.001$ for both). Also MPO levels in hepatic tissue were significantly lower in Group 3 than those in Group 2 ($P < 0.01$). However, TAS, OSI and MPO levels in hepatic tissue were significantly higher in Group 2 than those in Group 1 ($P < 0.01$ for all). There were not statistically significant differences between the *Nigella sativa* treatment group and the sham group regarding to the oxidant and antioxidant parameters ($P > 0.05$). The results are summarized in Table 1.

In histopathological evaluation, there were no pathological changes in liver tissue of the sham group (Figure 1A). Liver specimens from rats after ischemia-reperfusion exhibited focal necrosis and infiltration of leukocytes (Figure 1B). *Nigella sativa* treatment significantly decreased these pathological changes (Figure 1C). Histological tissue damage was milder in the *Nigella sativa* treatment group than that in the control group.

Nigella sativa did not produce any adverse side effects in the doses tested in our study.

DISCUSSION

An excessive production of oxygen free radicals has been reported in ischemic reperfused liver, leading to tissue damage, and this is an unavoidable process in liver transplantation and in the surgical procedures in which the Pringle maneuver is used^[4]. It has been shown in many studies that supplementation of free

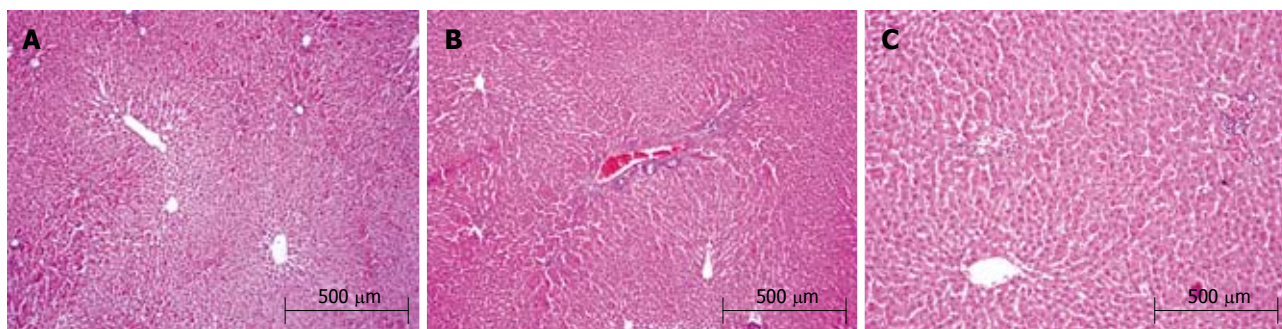


Figure 1 A: Normal liver tissue; B: Histopathological findings 60 min after reperfusion in the control group; C: Histopathological findings 60 min after reperfusion in the *Nigella sativa* treatment group.

radical scavengers is helpful in reducing hepatic ischemia reperfusion induced tissue damage^[4,6,8]. *Nigella sativa* has been identified as a potent antioxidant acting as a free radical scavenger^[9]. Therefore, it should not be surprising that *Nigella sativa* pretreatment has a protective effect on hepatic ischemia reperfusion injury in rats.

In some studies, to reduce the intestinal ischemia reperfusion injury, agents are administrated only before ischemia or reperfusion, and in some other studies both before ischemia and reperfusion. We preferred to administer both before ischemia and reperfusion as reported previously^[31].

In the present study *Nigella sativa* treatment markedly attenuated ALT, AST and LDH activities which are associated with hepatic parenchymal injury. The increase of AST, ALT and LDH activities observed in control groups can be elucidated by lipid peroxidation leading to cytolysis, which is caused by the oxygen free radical formed during the reperfusion phase^[32]. The decrease of AST, ALT, and LDH activities observed in the rats treated with *Nigella sativa*, when compared to the rats in control group, suggests a possible protective effect of *Nigella sativa* treatment in the hepatic ischemia/reperfusion condition.

Despite determination of either oxidants or antioxidant components alone may give information about oxidative stress, determination of oxidants along with antioxidants is more useful in this context^[33]. So, we preferred to measure oxidants and antioxidant capacity simultaneously to assess oxidative stress more exactly. In the present study, we measured oxidative stress with OSI which was detected using both oxidative and antioxidative parameters. We evaluated TAC which reflects the antioxidative status and TOS to investigate oxidative status using a more recently developed measurement methods by Erel^[27,28]. *Nigella sativa* treatment significantly reduced OSI and TOS levels, which show oxidative stress, and increased TAC levels, which show antioxidant capacity, in liver tissue. Oxidative stress activates mechanisms that lead to the synthesis of proinflammatory cytokines and cell adhesion molecules. Therefore, oxidative stress may contribute to an inflammatory response induced by endotoxemia after hepatic ischemia reperfusion. Our data confirm that liver ischemia reperfusion increases oxidative stress, an effect that not only produces direct

tissue damage, but also modulates production of toxic cytokines leading to inflammation and leukocyte infiltration, consistent with previous studies. In addition to this, *Nigella sativa* treatment alleviated pathological structural changes.

Infiltration of neutrophils into tissues is commonly assessed by changes in activity of MPO, which is an enzyme found primarily in neutrophils. Increased MPO activity in the liver of rats after I/R suggests activation of an inflammatory response. In our study, we observed increased MPO activity in the liver tissue, and this may indicate that neutrophil accumulation and lipid peroxidation contributes to ischemia reperfusion-induced liver injury. Previously, it has been reported that the activated neutrophils located in the inflammatory foci and secreting MPO into the extracellular space can convert hydroperoxides into free radicals, triggering lipid peroxidation^[34]. This is consistent with the results of our present study.

Catalase is an oxidoreductase enzyme, which transforms H_2O_2 into H_2O and O_2 . It can protect cells from damage induced by ischemia reperfusion through scavenging reactive oxygen species^[8]. The results of the present study showed that treatment with *Nigella sativa* can increase catalase activity, and this is consistent with its protective effect.

Although preliminary, our data indicate that *Nigella sativa* exhibits protective effects on liver tissue against ischemia reperfusion injury. The results of this present study may have clinical applications to the liver surgery associated with the Pringle maneuver and hepatic transplantation. However, more investigations are required to evaluate the protective effects of *Nigella sativa* on liver tissue damage in clinical and experimental models to verify this conclusion.

COMMENTS

Background

Hepatic ischemia-reperfusion (I/R) injury may occur in a variety of clinical settings such as trauma, sepsis, liver transplantation or hepatic pedicle clamping during a liver surgery and this remains a significant problem. Oxygen free radicals, produced on reperfusion have been shown to play a major role in hepatic I/R injury. Various therapeutic effects have been described for *Nigella sativa*. Additionally, it has been presented that *Nigella sativa* has protective effect against ischemia reperfusion injury to various organs. Therefore, it seems pos-

sible that the administration of *Nigella sativa* might protect the liver against the ischemia reperfusion injury and thus, we aimed to confirm this hypothesis.

Research frontiers

Tissue ischemia initiates a series of events that can ultimately lead to cellular dysfunction and necrosis. But, resumption of blood flow can paradoxically create more tissue injury, possibly because of the production of oxygen-derived cytotoxic products. It is important to reduce the oxidative stress mechanism and protect the liver tissue against the ischemia and reperfusion injury. This study demonstrated that *Nigella sativa* exhibits protective effects on liver tissue against ischemia reperfusion injury.

Innovations and breakthroughs

In previous studies, it has been demonstrated that *Nigella sativa* has protective effects against ischemia reperfusion injury on various organs. However, its protective effects on liver tissue against ischemia reperfusion injury are unclear.

Applications

It seems possible that the administration of *Nigella sativa* might protect the liver against the ischemia reperfusion injury which can occur due to a liver surgery, trauma or sepsis. Future studies will be required to verify the effectiveness of this substance.

Terminology

Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function.

Peer review

In the present study authors investigated the effects of *Nigella sativa*, an annual flowering plant, on hepatic ischemia-reperfusion injury. The late effects of *Nigella sativa* should be evaluated and survival experiments would be helpful.

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RAPID COMMUNICATION

Efficacy and safety of gemcitabine-oxaliplatin combined with huachansu in patients with advanced gallbladder carcinoma

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Abstract

AIM: To evaluate the efficacy and safety of gemcitabine-oxaliplatin (GEMOX) combined with huachansu (cinobufagin) injection treatment in patients with locally advanced or metastatic gallbladder carcinoma (GBC), and to assess the quality of life (QOL) of such patients.

METHODS: Twenty-five patients with locally advanced or metastatic GBC were treated with intravenous gemcitabine (1000 mg/m²) over 30 min on days 1 and 8, 2 h infusion of oxaliplatin (120 mg/m²) on day 1, and 2-3 h infusion of huachansu (20 mL/m²) on days -3-11, every 3-4 wk. Treatment was continued until occurrence of unacceptable toxicity or disease progression. QOL of patients was assessed by the EORTC QLQ-C30 at baseline, at the end of the first, third and sixth chemotherapy cycles, and 1 mo after the treatment.

RESULTS: Among the 25 patients with a median age of 64 years (range 42-78 years), 23 were evaluable in the study. A total of 137 cycles of therapy were performed and the median cycle was 5 (range 1-8) per patient. Out of the 23 patients whose response could

be evaluated, 8 partial responses (PR) were observed (34.8%), while 7 patients (30.4%) demonstrated a stable disease (SD). The disease control rate was 65.2%. Progression of cancer was observed in 8 (34.8%) patients. The median progression-free and overall survival time was 5.8 mo (95% CI: 4.5-7.1 mo) and 10.5 mo, respectively. The therapy was well tolerated, with moderate myelosuppression as the main toxicity. Anemia grade 2 was seen in 16.0%, neutropenia grade 3 in 8.0% and thrombocytopenia grade 3 in 24.0% of patients, respectively. Non-hematologic toxicity ranged from mild to moderate. No death occurred due to toxicity. The QOL of patients was improved after chemotherapy, and the scores of QOL were increased by 10 to 20 points.

CONCLUSION: GEMOX combined with huachansu (cinobufagin) injection is well tolerated, effective, thus improving the QOL of patients with advanced GBC.

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Key words: Gallbladder carcinoma; Gemcitabine; Oxaliplatin; Huachansu injection; Quality of life

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INTRODUCTION

There is a marked worldwide geographic variation in gallbladder carcinoma (GBC) incidence, which correlates with the prevalence of cholelithiasis, and the highest prevalence of GBC is in Israel, Mexico, Chile, Japan, and America^[1,2]. The incidence and mortality of GBC in China has had a tendency to increase in recent years^[3].

The early symptoms of GBC are similar to those of other gallbladder diseases, such as gallstones or infection.

But, no characteristic symptoms could be observed at its early stage. In fact, early gallbladder cancer is discovered often when the gallbladder is removed as a treatment for gallstones. Otherwise, gallbladder cancer is often at its advanced stage at the time when it is diagnosed, and has a postoperative 5-year survival rate of less than 5%^[4] with a high relapse rate.

Conventional surgery is considered the most effective treatment for GBC. But, many cases are inoperable at the time of its diagnosis. Chemotherapy has recently shown its effect on gallbladder cancer. The most commonly used chemotherapeutic agent is 5-fluorouracil (5-FU), which is often used alone or in combination with leucovorin. Several small trials of combined regimens for GBC, using 5-FU, cisplatin, mitomycin and/or leucovorin, have been reported to have mixed results. Other chemotherapeutic agents that are now in clinical trials include capecitabine, oxaliplatin^[5], gemcitabine^[6-8], erlotinib^[9], *etc.* In addition, more and more people have paid their close attention to Chinese medicines for the prevention and treatment of cancer. Huachansu (cinobufagin) is just a widely used antitumor agent of traditional Chinese medicine in China^[10,11].

Because the clinical data have demonstrated the effectiveness of gemcitabine on pancreatic cancer, and gallbladder shares a common embryological origin with the exocrine pancreas, we used gemcitabine-oxaliplatin (GEMOX) combined with huachansu injection (cinobufagin) in treatment of advanced GBC.

Since the prognosis of GBC patients is usually poor, it is important to maintain their health-related quality of life (HRQOL). The QOL of patients has been an important endpoint in assessment of GBC treatment. However, to our knowledge, only a few studies discussing QOL of patients with GBC, and there is no report on GEMOX and huachansu regimen for GBC patients. The aim of this study was to evaluate the efficacy and safety of GEMOX plus huachansu treatment of GBC, and to assess the QOL of GBC patients.

MATERIALS AND METHODS

Patients and eligibility criteria

From January 2003 to July 2005, of the 25 enrolled patients (10 males and 15 females) with locally advanced or metastatic GBC, 23 were assessable. The patients were required to have histologically confirmed diagnosis, measurable computed tomography (CT) scan or magnetic resonance imaging (MRI), Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0-2, age < 78 years, a life expectancy > 3 mo. No patient underwent anticancer procedures within 1 mo before the present study. The laboratory criteria were leukocyte count $\geq 4.0 \times 10^9/L$, neutrophils $\geq 1.5 \times 10^9/L$, platelet count $\geq 100 \times 10^9/L$, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) $\leq 2.5 \times$ upper limit of normal (ULN), serum

Table 1 Characteristics of patients with locally advanced or metastatic GBC before treatment

Characteristic	Data
Total No. of patients (evaluable for response)	25 (23)
Sex (female/male)	15/10
Median age (range)	64 (42-78)
Nevin tumor stage	<i>n</i> (%)
III	4 (16.0)
IV	12 (48.0)
V	9 (36.0)
ECOG performance status at baseline	
0	3 (12.0)
1	17 (68.0)
2	5 (20.0)
Site of metastasis	
Liver	7 (28.0)
Lung	2 (8.0)
Lymph nodes	12 (48.0)
Peritoneum	3 (12.0)
Bone	2 (8.0)
Recurrence after surgical resection	5 (20.0)
Pre-treatment requiring stent or percutaneous transhepatic drainage because of obstructive jaundice,	4 (16.0)
Pre-treatment chemotherapy	5-FU-based chemotherapy
Liver and renal functions	
Total bilirubin (6-20.5 $\mu\text{mol/L}$)	
Median	18
Range	7-58
Gamma-glutamyltransferase (183.4-833.5 U/L)	
Median	1033.5
Range	283.4-2583.9
Aspartate aminotransferase/Alanine aminotransferase (0-666.8 U/L)	
Median	950.2
Range	383.4-1600.3
Blood urea nitrogen (3.2-7.0 mmol/L)	
Median	6.2
Range	3.6-7.8
Serum creatinine (44-140 $\mu\text{mol/L}$)	
Median	78
Range	48-121
CA19-9 (0-39 U/L)	
Median	496
Range	7-10463

¹Normal level.

creatinine < $1.5 \times$ ULN, serum bilirubin value < $2.5 \times$ ULN. Basic CA19-9 level was recorded. Patients with active infections, unstable cardiovascular conditions, brain metastases, other malignancy or serious medical illnesses were excluded from this study. The pre-treatment characteristics are listed in Table 1.

Tumor size was assessed by CT scan or MRI within 3 wk prior to the first cycle, and baseline biological analysis was performed within 1 wk. A physical examination and complete blood cell count were performed 2-3 d before each cycle. Blood count was obtained each week to determine the level of myelosuppression. After every three cycles, a full clinical evaluation including performance status and physical examination was performed.

Treatment protocol

All patients received intravenous gemcitabine (1000 mg/m²) over 30 min on days 1 and 8, 2 h oxaliplatin infusion (120 mg/m²) on day 1 (oxaliplatin was discontinued if specific cumulative peripheral sensory neuropathy of NCI CTC grade 3 occurred), and 2-3 h huachansu infusion (20 mL/m²) (0.5 g/mL) in 500 mL of 50 g/L glucose solution on days -3-11. Treatment was repeated every 3-4 wk until limiting toxicity or disease progression occurred, or further treatments were refused by patients. Biliary bypass or stenting was required in four patients before the treatment.

Follow-up

After two cycles of chemotherapy, patients were followed up every 4-6 wk till February 2007.

Assessment of efficacy

Tumor response was evaluated every three cycles by CT scan or MRI, using standard RECIST criteria^[12]. Complete response (CR) was defined as a disappearance of all signs and symptoms of disease. Partial response (PR) was defined as a decrease > 30% of the sum of the largest diameters of target (measurable) lesions without appearance of new lesions or progression of non-target (evaluable) lesions. To be assigned a response status, changes in tumor measurement were confirmed by a repeated assessment performed no less than 4 wk after the criteria for response were first met. Stable disease (SD) was defined as no sufficient shrinkage to qualify partial response or less than a 20% increase in the sum of the largest diameters of target lesions without appearance of new lesions or progression of non-target lesions. Progressive disease (PD) was defined as a 20% increase in the sum of the largest diameters of target lesions or as appearance of new lesions or as progression of non-target lesions. Disease control was defined as the absence of tumor progression (i.e. complete and partial response and SD) for at least 2 mo. Progression-free survival (PFS) was determined from the first day of treatment until clinical progression or tumor progression assessed by CT scan. Overall survival (OS) was determined from the first day of treatment until the date of death. OS and PFS were analyzed using the Kaplan-Meier method. Toxicity was evaluated at each cycle according to the NCI CTC version 2.0.

Assessment of QOL

The validated traditional Chinese version of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire-Core 30 (EORTC QLQ-C30) was used to measure the patients' QOL. Patients completed the EORTC QLQ-C30 at baseline, at the end of the first, third and sixth chemotherapeutic cycles, and 1 mo after completion of chemotherapy.

The QLQ-C30 is a 30-item standardized measure that includes a global QOL/ overall health status scale, five functioning scales (physical, role, emotional, cognitive, and social), three multi-item symptom scales (fatigue,

pain, nausea and vomiting) and six single items (dyspnea, sleep disturbance, appetite loss, diarrhea, constipation, and financial impact). The raw scores obtained from the EORTC questionnaire were converted to scores ranging from 0 to 100 using linear transformation according to the scoring procedure. Higher scores on the functioning scales, and the overall health status indicate a higher functioning level and a better QOL. Higher scores on the symptom scales or single item scales represent a higher level of symptoms or problems.

QOL scores were determined in our study in the following domains: physical, role, emotional, cognitive and social function, global QOL/overall health status, fatigue, nausea and vomiting, and pain.

Statistical analysis

The objective response rate, progression-free survival, overall survival, toxicity and QOL were observed, and the 95% confidence interval (CI) was calculated using the method of Clopper and Pearson. Overall survival, progression-free survival, death or last follow-up was made using the Kaplan-Meier method using SPSS version 12.0 software. The life table method was used to evaluate the 1-year survival rate. Data were expressed as mean ± SD. QOL score analysis was done using the paired *t*-test for comparison between baseline and 1 mo after completing chemotherapy. *P* < 0.05 was considered statistically significant, whereas a mean difference of 10 or more points in QOL scales represents a clinically significant/relevant difference^[13].

RESULTS

Response and survival

A total of 25 patients (10 males and 15 females) were included this trial and 23 were assessable. Twenty-one patients (84.0%) received GEMOX plus huachansu injection as the first-line and 4 (16.0%) as the second-line chemotherapy. A total of 137 cycles of therapy were performed, the median cycle was 5 (range 1-8) per patient. Up to February 2007, two patients were still alive. The median follow-up time was 11.5 (range, 2-33) mo. The one-year survival rate was 39.1%. There was no complete response. However, out of the 23 patients whose response could be evaluated, 8 (34.8%) went into PR, 7 (30.4%) demonstrated SD, 8 (34.8%) had progression of the disease. The disease control rate was 65.2%. The median progression-free and overall survival was 5.8 mo (95% CI: 4.5-7.1 mo) and 10.5 mo, respectively (Figure 1).

Four patients (16.0%) required biliary tract decompression by endoscopic or percutaneous stenting because of obstructive jaundice before chemotherapy.

Basic CA19-9 levels were recorded in all patients (median 496; range 7-10 463 kU/L), and 15 patients (60%) had elevated baseline levels. Serum CA19-9 levels were measured every 3-4 wk. The relative changes of CA19-9 during chemotherapy was negatively correlated with the baseline and progression-free survival (*P* = 0.03).

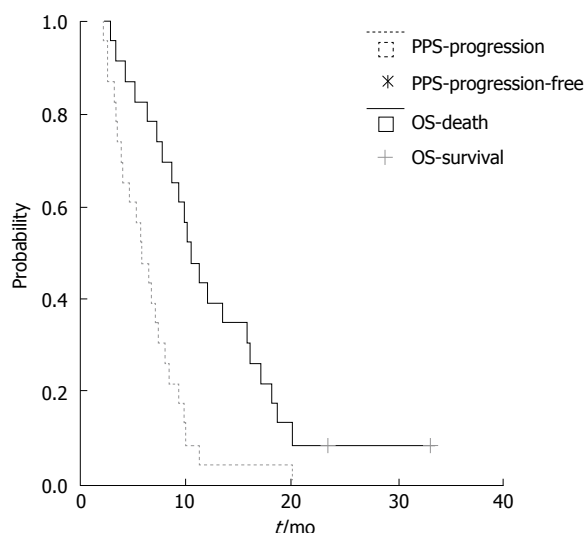


Figure 1 Kaplan-Meier curve of OS and PFS for patients treated with GEMOX plus huachansu. The median progression-free and median overall survival was 5.8 mo and 10.5 mo, respectively.

Toxicity

All patients were evaluable for toxicity. Gemcitabine in combination with oxaliplatin induced neutropenia, thrombocytopenia, nausea/vomiting, and peripheral neuropathy due their toxicity. It was observed by us that gemcitabine-based therapy was associated with a higher rate of thrombocytopenia among Chinese patients with cancer. Myelosuppression was the most frequent side-effect in our study. Anemia grade 2, neutropenia grade 3 and thrombocytopenia grade 3 were observed in 16%, 8% and 24% of patients, respectively. The nadir of thrombocytes appeared on days 6-12 [$(51.0 \pm 19.4) \times 10^9/L$, mean \pm SD]. Recombinant human interleukin-11 (IL-11) was used to treat chemotherapy-induced thrombocytopenia. The platelet count returned to normal [$(126.0 \pm 18.2) \times 10^9/L$] after 3-8 d of IL-11 treatment at a dose of 25-50 $\mu g/kg$ per day (Figure 2).

The symptoms induced by grade 1 non-hematologic toxicity included nausea/emesis (20.0%), diarrhea (4.0%), pain (8.0%), and peripheral neuropathy (64.0%). Sixteen patients (64.0%) felt anaesthesia of hands and feet, which was mild and did not interfere with their functions.

GEMOX combined with huachansu injection regimen was well tolerated (Table 2) with moderate myelosuppression as the main toxicity. No treatment-related death occurred.

QOL

The completion rate of QOL questionnaire declined from 96.0% at baseline to 73.9%, 1 mo after completion of chemotherapy. The mean score and standard deviation for the EORTC QLQ-C30 are presented in Table 3. An improvement of more than 10 points was found in the global QOL, physical, cognitive and emotional functioning, whereas symptoms such as pain decreased more than 18 points after chemotherapy. One month after the completion of treatment, physical

Table 2 Combined chemotherapy-related toxicities to patients *n* (%)

Toxicity	NCI-CTC grade			
	1	2	3	4
Leukopenia	9 (36.0)	5 (20.0)	2 (8.0)	0
Neutropenia	6 (24.0)	7 (28.0)	2 (8.0)	0
Anemia	2 (8.0)	4 (16.0)	0	0
Thrombocytopenia	7 (28.0)	5 (20.0)	6 (24.0)	2 (8.0)
Diarrhea	1 (4.0)	0	0	0
Nausea/emesis	5 (20.0)	0	0	0
Peripheral neuropathy	16 (64.0)	0	0	0
Pain	2 (8.0)	0	0	0

NCI-CTC: National Cancer Institute Common Toxicity Criteria. The combined treatment induced myelosuppression.

Table 3 Transformed scores for EORTC QLQ-C30 at different time points in patients treated with GEMOX and huachansu injection (mean \pm SD)

Domain/Item	Baseline	First cycle	Third cycle	Sixth cycle	1 mo
Functioning					
Physical	65.4 \pm 17.0	57.0 \pm 20.3	64.4 \pm 21.0	70.6 \pm 18.3	77.5 \pm 16.4 ^a
Role	57.1 \pm 26.3	55.0 \pm 24.3	58.5 \pm 26.2	60.4 \pm 25.4	63.4 \pm 27.2
Emotional	54.3 \pm 33.0	47.2 \pm 36.8	59.4 \pm 39.1	70.5 \pm 24.2	69.4 \pm 30.1 ^a
Cognitive	66.1 \pm 24.6	68.1 \pm 30.2	77.4 \pm 26.3	79.1 \pm 24.0	79.5 \pm 21.7 ^a
Social	47.2 \pm 30.4	39.4 \pm 33.6	44.1 \pm 29.0	41.1 \pm 30.7	48.2 \pm 32.6
Global QOL	52.3 \pm 20.0	41.4 \pm 24.3	50.6 \pm 29.7	55.4 \pm 30.0	65.3 \pm 22.4 ^a
Symptoms					
Fatigue	43.3 \pm 31.2	51.2 \pm 33.4	40.1 \pm 27.4	42.1 \pm 22.0	38.2 \pm 28.6
Nausea and vomiting	7.6 \pm 6.2	9.0 \pm 5.1	7.4 \pm 4.3	5.4 \pm 2.5	3.2 \pm 2.1 ^a
Pain	44.5 \pm 21.6	28.0 \pm 18.3	25.5 \pm 12.4	24.3 \pm 9.1	26.0 \pm 10.4 ^a

Quality of life was assessed at baseline, at the end of the first, third and sixth chemotherapeutic cycles, and 1 mo after completion of chemotherapy. Scores of EORTC QLQ-C30 were converted to linear transformation ranging from 0 to 100. Higher scales for the functional and global QOL represent a better function. In contrast, higher scales for symptoms represent worse symptoms. ^a*P* < 0.05 *vs* baseline.

functioning (PF) increased over baseline levels by approximately 12 points, and the global QOL score increased from 52.3 ± 20.0 at baseline to 65.3 ± 22.4 , 1 mo after completion of chemotherapy. As compared with baseline, the patients reported fatigue that was worse immediately after the first cycle. However, fatigue then improved from the end of the first cycle to 1 mo after treatment. The five functional scales, global QOL, and multi-item symptom scales are shown in Figure 3. One month after treatment, the trend to improve the functioning, global QOL and symptoms was significant.

DISCUSSION

Due to the high mortality rate of GBC in China, we made this study to test the efficacy and safety of GEMOX combined with huachansu injection (cinobufagin) in patients with locally advanced or metastatic GBC and to assess the patients' QOL.

GBC, though rare, has a very poor prognosis. There is no generally accepted standard chemotherapy for

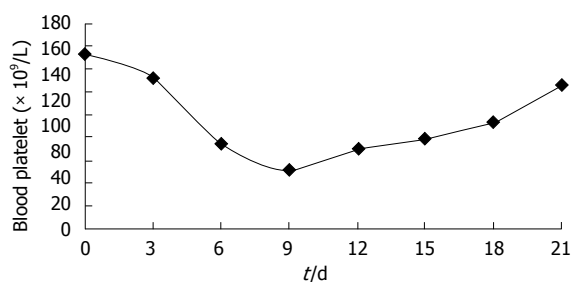


Figure 2 Peripheral blood platelet count after combined chemotherapy. The nadir of thrombocytes appeared on days 6-12.

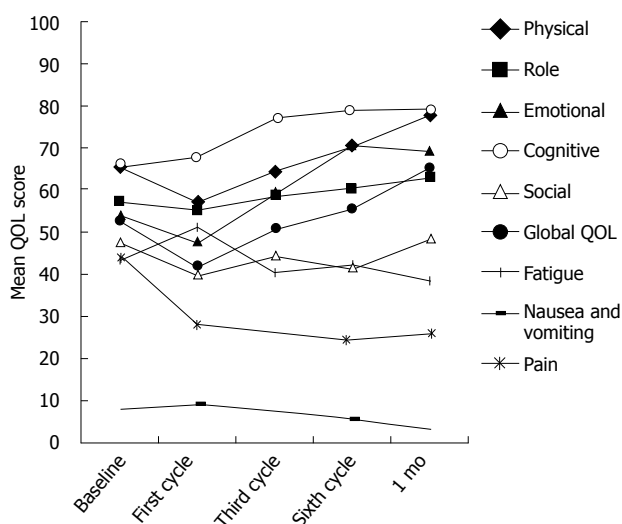


Figure 3 Functioning and symptom scales of the EORTC QLQ-30. First, third and sixth cycles: At the end of the first, third and sixth chemotherapeutic cycles; 1 mo: 1 mo after completion of chemotherapy. An improvement of more than 10 points was found in global QOL, physical and cognitive as well as emotional functioning, whereas symptoms such as pain were decreased by more than 18 points after completion of chemotherapy.

advanced, non-resectable cancer of the gallbladder or biliary tree. The median survival time of advanced GBC patients who receive best supportive care is approximately 6 mo^[14].

Some new chemotherapeutic agents have been used in treatment of patients with advanced biliary tract cancer recently, such as gemcitabine, 5-fluorouracil^[15-17], capecitabine^[18], cisplatin^[19,20], oxaliplatin or carboplatin. It was reported that gemcitabine is active against pancreatic and advanced biliary tract adenocarcinoma, and able to induce a response rate of 8.0%-60.0%^[21,22].

Gallardo^[23] conducted a phase II trial with gemcitabine (1000 mg/m² over 30 min weekly for 3 wk followed by a week of rest) in patients with locally advanced or metastatic GBC, and found that the overall response rate was 36.0%. The cancer remained stable in 6 (25.0%) patients, and progressed in 10 (40.0%) patients. The median survival time was 30 wk. In another phase II trial with 24-h gemcitabine infusion weekly in patients with advanced gallbladder and biliary tract carcinoma, 18 patients were evaluated for response. One partial response was observed (6.0%), the disease control rate was 61.0%, the median time of tumor progression

was 3.6 mo and the median overall survival time was 7.5 mo^[24].

Andre *et al*^[25] used GEMOX regimen to treat patients with advanced biliary tract adenocarcinoma, and found that the objective response was 36%, the median PFS time was 5.7 mo, the overall survival time was 15.4 mo in group A (PS 0-2), while the objective response was 22.0%, the median PFS time was 3.9 mo and the median OS time was 7.6 mo in group B (PS > 2).

Huachansu, one of the most widely studied traditional Chinese medicine, is a water soluble extract from *Bufo* toad skin, and can be used in treatment of cancer, especially liver and pancreatic cancer. Some studies demonstrated that huachansu injection can improve the QOL of patients and has been used as a treatment of cancer in China^[26-28]. It was reported that digitalis-like cinobufagin can protect prostate cancer cells from proliferation. The protein expression of active caspase 3 in LNCaP, DU145, and PC3 cells was increased after treated with combined cinobufagin and EGTA, the expression of Fas was increased, the expression of Bax was down-regulated in nuclei, and the protein expression of cytosolic cytochrome C was also increased after treatment with cinobufagin in these cell lines^[29]. It has been shown that bufalin or cinobufagin increases Ca²⁺ and apoptosis in cancer cells, caspase 3 activities in DU145 and PC3 cells, and caspase 9 activities in LNCaP cells after a 24 h culture^[30].

QOL is a multidimensional concept including physical, emotional, social, and daily-life functioning as well as disease symptoms and treatment from the patient's perspective. Improving QOL and disease-associated symptoms is increasingly important for patients with advanced GBC. QOL is now regarded as a biologically and clinically meaningful outcome that is as important as disease-free, and overall survival with regard to anticancer treatment. Huachansu injection was given 3 d before GEMOX in our study, and its effectiveness on pain relief appeared 1-2 d later (9/14 patients). The overall life quality of patients entered this trial improved 10 to 20 points. The patients undergoing GEMOX and huachansu regimen achieved better QOL outcomes. The partial response rate was 34.8%, and the disease control rate was 65.2%. The median progression-free and median overall survival was 5.8 mo and 10.5 mo, respectively. The patients tolerated the treatment well with moderate myelosuppression as the main toxicity. No treatment-related deaths occurred.

In conclusion, combined GEMOX and huachansu injection regimen can be used in treatment of GBC patients, especially those with advanced or metastatic GBC.

COMMENTS

Background

The difficulty in early diagnosis of gallbladder carcinoma (GBC) is its poor specificity in clinical and ambiguous early symptoms, thus affecting its prognosis. Some patients are found having GBC only when other diseases are diagnosed and treated. Surgery is the only curative treatment for gallbladder

cancer. However, because of frequent local and distant recurrence, radical surgery at its advanced stage is often unsuccessful. Thus, chemotherapy for patients with advanced GBC seems to be a better choice of treatment. However, no standard chemotherapy for GBC has yet been established.

Research frontiers

The increasing number of papers on chemotherapy for GBC emphasizes the need of a new standard beyond 5-FU. At present, clinical studies on the treatment of GBC with new chemotherapeutic agents are underway. Further clinical trials, especially large multi-institutional RCTs (phase III studies) using novel agents such as gemcitabine, should be performed in order to establish a standard treatment for GBC.

Innovations and breakthroughs

In China, huachansu used in treatment of patients with lung cancer and hepatocellular carcinoma has achieved rather good results in suppressing the growth of cancer, alleviating of pain and fatigue, and improving the function of patients' immune system. Our findings provide certain possible evidence that combined gemcitabine-oxaliplatin (GEMOX) and huachansu chemotherapy may improve the survival and QOL of GBC patients.

Applications

The results of this study indicate that combined GEMOX and huachansu chemotherapy is an effective regimen for metastatic GBC. The patients can tolerate it well. Toxicities are mostly hematological and easily manageable. If this combined regimen could be applied in clinic practice, the patients would have a longer survival time.

Terminology

The World Health Organization (WHO) defines QOL as "an individual's perception of his or her position in life in the context of culture and value system in which he or she lives in relation to his or her goal, expectation, standard and concern. It is a broad-ranging concept affected in a complex way by the person's physical health, psychological state, independence, social relationships, and his or her relationship to salient feature of his or her environment." Studies showed that QOL is associated to the survival of cancer patients.

Peer review

This is an interesting study. The authors evaluated the efficacy and safety of combined GEMOX and huachansu injection (cinobufagin) treatment in patients with locally advanced or metastatic gallbladder carcinoma, and assessed the patients' QOL. The study was well designed.

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Efflux pump gene *hefA* of *Helicobacter pylori* plays an important role in multidrug resistance

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CONCLUSION: The efflux pump gene *hefA* plays an important role in multidrug resistance of *H. pylori*.

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Key words: Efflux pump; *Helicobacter pylori*; Multidrug resistance; Fluorescence real-time quantitative PCR; Knockout mutant

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Abstract

AIM: To determine whether efflux systems contribute to multidrug resistance of *H. pylori*.

METHODS: A chloramphenicol-induced multidrug resistance model of six susceptible *H. pylori* strains (5 isolates and *H. pylori* NCTC11637) was developed. Multidrug-resistant (MDR) strains were selected and the minimal inhibitory concentration (MIC) of erythromycin, metronidazole, penicillin G, tetracycline, and ciprofloxacin in multidrug resistant strains and their parent strains was determined by agar dilution tests. The level of mRNA expression of *hefA* was assessed by fluorescence real-time quantitative PCR. A *H. pylori* LZ1026 knockout mutant ($\Delta H. pylori$ LZ1026) for (putative) efflux protein was constructed by inserting the kanamycin resistance cassette from pEGFP-N2 into *hefA*, and its susceptibility profiles to 10 antibiotics were evaluated.

RESULTS: The MIC of six multidrug-resistant strains (including 5 clinical isolates and *H. pylori* NCTC11637) increased significantly (≥ 4 -fold) compared with their parent strains. The expression level of *hefA* gene was significantly higher in the MDR strains than in their parent strains ($P = 0.033$). A *H. pylori* LZ1026 mutant was successfully constructed and the $\Delta H. pylori$ LZ1026 was more susceptible to four of the 10 antibiotics. All the 20 strains displayed transcripts for *hefA* that confirmed the *in vitro* expression of these genes.

INTRODUCTION

H. pylori is a Gram-negative bacterium that colonizes human gastric mucosa^[1] and plays an important role in the pathogenesis of chronic gastritis, peptic ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma^[2,3]. Successful treatment usually requires two or three kinds of antibiotics in combination with a proton pump inhibitor. In recent years, the eradication rate of *H. pylori* for standard therapies has decreased due to the increasing antimicrobial resistance^[4,5]. Possible mechanisms of intrinsic drug resistance involve decreased drug uptake or increased drug efflux^[6]. Efflux of compounds is a phenomenon commonly observed in bacteria^[7-9]. Through this process, organisms are protected from possible toxic effects of metabolite accumulation or external compounds, and compound efflux results in a decreased susceptibility to a variety of antibiotics. Five families of multidrug efflux transporters have been described^[10]. One of them, widespread in Gram-negative bacteria, is the resistance-nodulation-division (RND) family of efflux systems^[11-13]. The RND family of efflux systems has three components: inner membrane efflux proteins (IEPs) which act with the other two components, a periplasmic efflux protein (PEP) which facilitates interaction with the other two components, and an outer membrane efflux protein (OEP) which

is the TolC (the outer membrane efflux protein in *Escherichia coli*) or a TolC homolog^[14].

Bina JE and coworkers^[15] have identified three RND efflux systems, namely *hefABC*, *hefDEF*, and *hefGHI*. Each of them is consisted a translocase, an accessory protein, and a TolC homolog. *hefA*, *hefD*, and *hefG* are the TolC homolog encoding the outer membrane efflux protein. In their study, the *hefGHI* operon was expressed only *in vivo*. Nevertheless, they could not establish a role of these efflux systems in antibiotic resistance. An unfortunate choice of compounds and a methodology that may not be sensitive enough to relatively small differences in susceptibilities between the mutant and parental strains may have impacted their study. van Amsterdam *et al*^[16] identified 27 putative translocases in the *H pylori* 26695 genome, but only four putative *H pylori* OEPs or TolC homologs. Parallel translocases may function in a limited number of OEPs^[17]. Thus, inactivation of a TolC-like protein may affect the functions of multiple translocases. They evaluated the susceptibility profiles of the inactivation of the four TolC homologs after insertion, but could not quantitate the operon expression levels of the efflux systems in multidrug resistant strains of *H pylori*. In view of the biological difference of the *H pylori* strains in Asia, Europe and North America^[18], the present study was to determine whether efflux systems of *H pylori* contribute to antimicrobial susceptibility. A chloramphenicol-induced multidrug resistance model of 6 susceptible *H pylori* strains (5 isolates and *H pylori* NCTC11637) was developed by detecting the minimal inhibitory concentration (MIC) of the strains to metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin. The levels of mRNA expression of *hefA* in multidrug-resistance (MDR) strains and their parent strains were assessed by fluorescence real-time quantitative PCR. To further evaluate the role of *hefA* in multidrug resistance of *H pylori*, a clinical isolate of *H pylori* LZ1026 was selected, which represent the Chinese strains. A *H pylori* LZ1026 knockout mutant was constructed and its susceptibility profiles to 10 kinds of antibiotics were evaluated.

MATERIALS AND METHODS

Reagents

Chemical reagents were purchased from TaKaRa Biotechnology Co. Ltd (Dalian, China). Restriction enzymes, polymerases, T4 DNA ligase, Quant SYBR Green PCR Kits and other molecular biology reagents were purchased from Biosail Biotechnology Co. Ltd (Beijing, China), and used following their manufacturers' instructions. Amoxicillin, cefotaxime, ceftazidime, polymyxin B, nalidixic acid, clarithromycin, norfloxacin, gentamicin, metronidazole, tetracycline, erythromycin, chloramphenicol, and ciprofloxacin, penicillin G were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Nucleic acid sequencing and synthesis were performed in SaiBaiSheng Biotechnology Co. Ltd (Beijing, China).

Bacterial strains, plasmid and culture conditions

H pylori LZ1026 and 20 other clinical isolates of *H pylori* were obtained from routine cultures of clinical gastric biopsies from patients with peptic ulcer or chronic active gastritis at the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). *H pylori* NCTC11637 obtained from HeNan Key Laboratory of Molecular Medicine was used as a reference. Isolates were cultured on Brucella agar medium plates containing 7% lysed sheep blood at 37°C under microaerobic conditions (50 mL/L O₂, 100 mL/L CO₂, 850 mL/L N₂) for 48-72 h. Identification of *H pylori* isolates was based on the results of Gram staining, cell morphology, and positive reaction for catalase, oxidase and urease activity. Putative multidrug efflux knockout mutant of *H pylori* LZ1026 was grown on sheep blood agar plates containing 25 µg of kanamycin (KAN) per mL and 100 µg of ampicillin (AMP) per mL. The vectors pBluescript II SK (-) and pEGFP-N2 were from HeNan Key Laboratory of Molecular Medicine. *E. coli* DH5α was used for subcloning the *hefA* operon. *E. coli* were grown in Luria broth. For cloning in *E. coli*, 100 mg/mL AMP was used.

Induction of multiple antibiotic resistances of clinical isolates

Susceptible strains were isolated from gastric biopsy samples using multiple antibiotic resistance induction tests. Induction of chloramphenicol resistance of susceptible isolates was performed by selecting resistant colonies that arose in the agar plates containing 1/2 × MIC chloramphenicol. The resistant colonies were incubated for 48-72 h under microaerobic conditions to increase the concentration of chloramphenicol by one fold up to 128 × MIC. The strains were further incubated on fresh plates with no chloramphenicol for 4 generations, and then transferred onto plates containing 4 × MIC chloramphenicol^[19].

Induced colonies were maintained on plates containing 4 × MIC of erythromycin, metronidazole, tetracycline, penicillin G or ciprofloxacin, respectively. The colonies were incubated for 48-72 h under microaerobic conditions. A four-fold increase in MIC of the multidrug resistant strains was considered significant^[20]. Six multidrug resistant strains were selected.

Assessment of susceptibilities to antimicrobials

H pylori cells grown for 48 h on sheep blood agar plates were resuspended in phosphate-buffered saline. Suspensions of *H pylori* were adjusted to an optical density of 0.1 at 625 nm, and 1 µL of these suspensions containing approximately 10⁵ CFU/mL, was spread on horse blood agar plates containing approximately 5 × 10⁴ bacteria within 5 mm^[21]. MICs of erythromycin, metronidazole, penicillin G, tetracycline, and ciprofloxacin in multidrug resistant strains and their parent isolates were determined with the conventional two-fold agar dilution tests. MICs of the kinds of 10 antibiotics against the *hefA* mutant strain of *H pylori* LZ1026 and its wild strain were also detected^[22].

Real-time RT-PCR

RNA was isolated using the total RNA kit (SBS Genetech Co., Ltd Beijing, China) and reverse transcribed into cDNA. Complete removal of DNA was verified by direct PCR with the RNA as a template (Figure 1). *hefA*, versus *gyrB* (a housekeeping gene encoding for gyrase B), was utilized to study the relative expression of the *hefA* gene in 6 multidrug resistant strains, and their parent strains: *H pylori* 03154, *H pylori* 12025, *H pylori* 12021, *H pylori* 11032, *H pylori* 03174, NCTC11637. cDNA of *hefA* and *gyrB* was amplified using a 5700 sequence detector real time PCR machine (Perkin Elmer Company) in the presence of Real Master Mix (SYBR Green). The gene-specific primers used were designed based on the sequence alignments of the genes from *H pylori* 11637 in GenBank. The sequences of *hefA* (accession No: AF059041) are F: (5'-ACGCCTCGAGTAAAAGCG CAAGGGAATTG-3') and R: (5'-ACGCTCTAG ATTTCGCTAATTGGCCTAGCAT-3'). The PCR primers were predicted to amplify a 162-bp amplicon. Expression of the housekeeping gene *gyrB* (accession No: AB084049) was assessed in parallel with the primer pair *gyrB*: F: (5'-TTACTACGACTTATCCTGGGGCTA GCGCTG-3') and R: (5'-CCCCATCAATTTCACAT TCTCCGC-3'). The PCR primers were predicted to amplify a 267-bp amplicon.

To plot standard curves for real-time PCR, cDNAs of the *hefA* and *gyrB* genes were cloned into the pMD19-T vector system (TaKaRa Biotechnology). The vector containing cDNAs of the *hefA* or *gyrB* genes with known molar concentrations was utilized. Single reactions were prepared for each cDNA along with each serial of dilution using the SYBR Green Master Mix (Stratagene). Each PCR also included a reverse transcription negative control to confirm the absence of genomic DNA, and a non template negative control to check the primer-dimer. Each reaction consisted of 20 μ L, containing 2 μ L of cDNA and 5 pmol of each primer. The cycling conditions were 1 cycle of denaturation at 95°C for 1 min, followed by 35 three-segment cycles of amplification at 95°C for 30 s, at 55°C for 1 min, and at 68°C for 30 s during which the fluorescence was automatically measured, and one three-segment cycle of product melting at 95°C for 1 min, at 55°C for 30 s, and at 95°C for 30 s. The baseline adjustment method of the Gene Amp 5700 SDS (Perkin-Elmer) software was used to determine Ct (the threshold cycle) in each reaction. A melting curve was plotted for each primer pair to verify the presence of one gene-specific peak, and the absence of primer dimer. All samples were amplified in three independent reactions, and the mean was used for further analysis^[23-25].

To assess whether the differences were actually statistically significant, an analysis of paired-samples *t* - test was utilized to study the degree of statistical significance of *hefA* gene expression between MDR and parent strains.

Mutagenesis of putative efflux operon

All standard DNA techniques, transformation of *E.coli*, and DNA analysis procedures were performed as

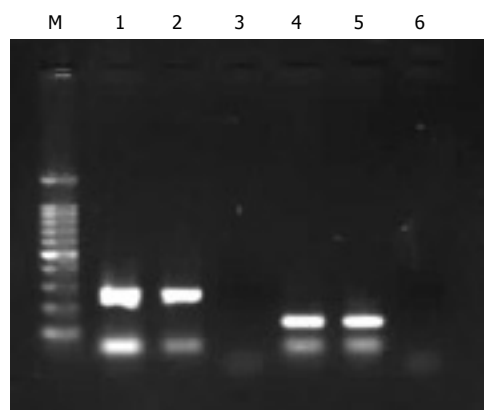


Figure 1 RT-PCR products of the *hefA* and *gyrB* genes. M: 100 bp DNA Marker; lanes 1 and 2: PCR products of *gyrB*; lanes 4 and 5: PCR products of *hefA*; lanes 3 and 6: PCR products with RNA as a template.

previously described^[26-28]. Plasmid DNA was isolated using a TIANprep Mini plasmid kit (TianGen Biotech Co. Ltd, Beijing, China). *H pylori* LZ1026 strain was isolated from a patient with peptic ulcer, and its internal portion (bp 277 to 1 574) of the *hefA* gene (reference from *H pylori* 11637 in GenBank accession number AF059041) was amplified. A restriction fragment, approximately 1298 bp, was purified and ligated into *Xba*I and *Xho*I-digested vector pBluescript II SK(-). *H pylori* LZ1026 knockout mutant for (putative) efflux protein was constructed by inserting the KAN resistance cassette from vector pEGFP-N2 into *hefA*, resulting in an 859 bp deletion. This suicide plasmid was then introduced into *H pylori* LZ1026 by natural transformation, and transformants were selected by plating them on a selective medium containing 25 mg of kanamycin/mL. Insertion of the KAN resistance cassette at the desired location(s) in the *H pylori* putative efflux gene was confirmed by PCR. Each clone was sequenced by Biosia Biotechnology Company (Shanghai, China).

RESULTS

Phenotypic induction of *hefA*-mediated resistance of *H pylori*

To obtain the multidrug-resistant strains, a chloramphenicol-induced model was developed. Following one-fold concentration increase of chloramphenicol, the MICs of chloramphenicol-induced strains resistant to metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin were detected. The MICs of 6 multidrug-resistant strains (including 5 clinical isolates and *H pylori* NCTC11637) were significantly increased (\geq 4-fold) compared with their parent strains (Table 1).

Expression of *hefA* in multidrug resistant strains (MDR) and their parent isolates (PT)

The expression of *hefA* in multidrug resistant strains and their parent isolates was assessed by relative real-time RT-PCR. Each relative expression value was the mean of three replicas. The relative expression of *hefA* versus *gyrB*

Table 1 Chloramphenicol-induced multidrug resistance profiles of *H pylori* strains

Strains	Exptl condition	Agar dilution test MIC ($\mu\text{g/mL}$)				
		MTZ	ERY	CIP	TET	PEN
03154	Before induction	0.125	0.125	0.25	0.25	0.125
	After induction (fold)	8×	4×	4×	8×	16×
12025	Before induction	2	0.063	0.125	0.03	0.063
	After induction (fold)	16×	2×	8×	4×	16×
12021	Before induction	4	0.125	0.25	0.125	0.063
	After induction (fold)	16	1×	16×	8×	8×
11032	Before induction	1	0.063	0.063	0.063	0.25
	After induction (fold)	8×	4×	4×	16×	16×
03174	Before induction	0.5	0.125	0.25	0.125	0.125
	After induction (fold)	8×	1×	8×	4×	32×
11637	Before induction	0.5	0.063	0.125	0.03	0.25
	After induction (fold)	4×	2×	4×	8×	16×

MTZ: Metronidazole; ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; PEN: Penicillin G.

Table 2 Relative expression of *hefA* in PT and MDR strains

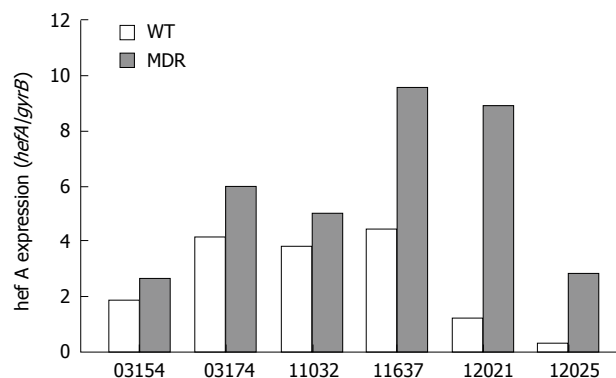
Strains	PT (<i>hefA</i> / <i>gyrB</i>)	MDR (<i>hefA</i> / <i>gyrB</i>)
03154	1.87	2.67
12025	0.32	2.84
12021	1.22	8.94
11032	3.84	5.04
03174	4.16	6.03
11637	4.41	9.56
Total (mean \pm SD)	2.6356 \pm 1.7245	5.8466 \pm 2.9370

PT: Parent isolates; MDR: Multidrug resistant strains.

in 5 clinical isolates and *H pylori* 11637 was significantly higher in MDR (5.8466 \pm 2.9370) than in PT (2.6356 \pm 1.7245) (Table 2). The results reveal that the relative expression of *hefA* was higher in the induced multidrug resistant strains than in their wild isolates. The difference in *hefA* expression was statistically significant ($P = 0.033$) (Figure 2).

Active efflux systems in *H pylori*

To further evaluate the role of efflux pump gene *hefA* of *H pylori* in multidrug resistance, a *H pylori* LZ1026 mutant was successfully constructed, and its disrupted *hefA* gene was uniquely different from that of the wild strain. A *hefA* gene fragment (about 1147 bp) of *H pylori* LZ1026 was sequenced and submitted to the GenBank (accession No. EU271683). Susceptibilities to wild-type strain of *H pylori* (*H pylori* LZ1026) and its isogenic mutant with putative RND outer membrane efflux protein were determined. The *hefA* (*H pylori* 0605, numbers refer to the loci in *H pylori* 26695) was genetically inactivated. MICs were determined by agar dilution tests. The *H pylori* mutant increased its susceptibility. The MIC of four of the 10 tested kinds of antibiotics (amoxicillin, cefotaxime, ceftazidime, polymyxin B, ciprofloxacin, nalidixic acid, clarithromycin, norfloxacin, chloramphenicol, and gentamicin,) was decreased at least 8-fold in the *H pylori* LZ1026 mutant, 8-fold in clarithromycin, 32-fold in cefotaxime, 16-fold

**Figure 2** Expression of the *hefA* gene in PT and MDR strains.

in chloramphenicol, 8-fold in gentamicin. The MICs were decreased after treatment with different antibiotics, which is consistent with the hypothesis that the product of *hefA* participates in multidrug efflux.

Transcribed gene encoding TolC homolog in vitro

Transcription of the gene *hefA* encoding a homolog of *E. coli* TolC outer membrane protein was assessed by RT-PCR with cDNA obtained from 20 different *H pylori* isolates. All the 20 strains displayed transcripts of *hefA* that confirmed the *in vitro* expression of these genes (data not shown).

DISCUSSION

Treatment of *H pylori* infection usually requires administration of two or more kinds of antibiotics concurrently. However, it may ultimately not be sufficient due to increased antibiotic resistance. Indeed, bacterial resistance to antibiotics hampers treatment of *H pylori* infections. Besides chromosomally-encoded drug resistance, intrinsic resistance to toxic compounds through increased export might be of importance in multidrug resistance of *H pylori*. Efflux systems have been identified in *H pylori*^[15,16], yet the possibility that these systems are implicated in multidrug resistance has not been established previously^[15]. In this study, the *hefA* gene was chosen from three TolC homologs: *hefA*, *hefD* and *hefG*, which encode the outer membrane efflux protein in *H pylori*. It was demonstrated earlier that the *hefD* and *hefG* genes are only poorly expressed *in vitro* and only RND efflux systems are known to have a wide variety of antibiotic substrates^[15,22].

In the present study, because it was laborious to select the multidrug resistant strains from clinical isolates of *H pylori*, we developed a chloramphenicol-induced multidrug resistant model of *H pylori*, for the first time to our knowledge. Using this model, six multidrug resistant strains were elicited successfully, and their MICs of metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin were significantly increased (≥ 4 -fold) compared with their parent strains, except that the MIC of erythromycin was mildly increased in 4 wild strains, indicating a difference in substrate specificity with other bacteria^[29,30]. In contrast, they could only dampen the

function of a group of efflux systems by constructing mutant strain. But, they could not quantitate the operon expression levels in the efflux systems^[16]. In this study, the relative real-time RT-PCR was introduced to assess the expression level of the *hefA* gene encoding the outer membrane efflux protein in 5 clinical isolates and *H pylori*11637, suggesting that the relative expression of *hefA* is higher in induced multidrug resistant strains than in their wild susceptible isolates. This is consistent with the hypothesis that the expression of RND efflux systems in many bacteria is regulated by environmental stimuli, including the presence of antibiotics and antimicrobial compounds, growth stage, and stress factors^[7-9]. By inserting the KAN resistance cassette into *hefA*, we also constructed a *H pylori* mutant strain ($\Delta H pylori$ LZ1026). By detecting the susceptibility to 10 kinds of antimicrobials with a more sensitive method of two-fold agar dilution tests, we showed that similar to other gram-negative organisms, the *H pylori hefA* mutant increased its susceptibility to antibiotics, which is consistent with the findings in a previous study^[22], confirming that high expression of the *hefA* plays an important role in MDR of *H pylori*. Since *H pylori* contain an active multidrug efflux mechanism, compound efflux needs to be taken into account when determining resistance mechanisms in *H pylori*.

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COMMENTS

Background

In recent years, the eradication rates of *H pylori* for standard therapies have decreased due to increasing antimicrobial resistance. Multidrug resistant *H pylori* strains have already been discovered. Possible mechanisms underlying multidrug resistant *H pylori* strains are involved in decreased drug uptake or increased drug efflux. Efflux of compounds is a phenomenon commonly observed in bacteria, and compound efflux decreases the susceptibility of *H pylori* strains to a variety of antibiotics. The contribution of efflux proteins to antibiotic resistance of *H pylori* strains is not well established.

Research frontiers

With the increasing antimicrobial resistance to *H pylori*, multidrug resistant *H pylori* strains have been discovered. Researches on the mechanisms of antibiotic resistance of *H pylori* have becoming a medical research hotspot. The mechanism underlying multidrug resistance of *H pylori* is still unclear.

Innovations and breakthroughs

The contribution of efflux proteins to antibiotic resistance of *H pylori*, strains is not well established. In view of the biological difference of *H pylori* strains in Asia, Europe and North America, *H pylori* strains were isolated from Chinese people in the present study. The study was to determine whether efflux systems in *H pylori* strains contribute to their antimicrobial susceptibility. A chloramphenicol-induced multidrug resistant model of *H pylori* strains was developed. The level of mRNA expression of *hefA* in multidrug-resistant (MDR) strains and their parent strains were assessed by fluorescence real-time quantitative PCR. A *H pylori* LZ1026 knockout mutant was constructed and its susceptibility profiles to 10 kinds of antibiotics were evaluated.

Applications

This study confirmed that high expression of the *hefA* plays an important role in MDR of *H pylori*. Since *H pylori* contain an active multidrug efflux mechanism,

compound efflux needs to be taken into account when determining resistance mechanisms in *H pylori*.

Terminology

Efflux pump is chromosomally located or acquired by bacteria, which can either be activated by environmental signals or by a mutation in a regulatory gene. Two major categories exist: one is energized by proton motive force and the other is dependent on ATP. The pumps may have limited or broad substrates, the so-called multiple drug resistant pumps themselves form a number of related families.

Peer review

The authors studied the significance of efflux pump gene *hefA* in multidrug resistance of *H pylori*. The study is interesting and well designed.

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Combined endoscopic and ursodeoxycholic acid treatment of biliary cast syndrome in a non-transplant patient

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INTRODUCTION

Though uncommon, biliary casts are most frequently described in liver transplant patients^[1-4]. The etiology of cast development is not fully known, but it is likely to be multifactorial with biliary sludge being a prerequisite for cast formation. Bile duct damage and ischemia, hemolysis, cholangitis, fasting, total parenteral nutrition (TPN) and recent surgery are thought to be implicated in cast, pathogenesis *via* sludge development^[3,4].

We describe a diabetic man who developed a biliary cast after cholecystectomy. The patient underwent endoscopic retrograde cholangiopancreatography (ERCP) with extraction of an extrahepatic biliary cast. But, his cholestasis remained unchanged. Oral administration of ursodeoxycholic acid (UDCA) resulted in normalization of liver function tests.

CASE REPORT

A 76-year-old diabetic patient underwent emergent cholecystectomy for gangrenous calculous cholecystitis. He had a protracted postoperative course complicated with *Candida* esophagitis treated with 200 mg fluconazole, twice per day and TPN for 15 d during which dyslipidemia was present. His cholesterol level was 503 mg/dL (< 200 mg/dL) and triglyceride was 967 mg/dL (< 200 mg/dL). Seven weeks later, he presented with abdominal pain, chills and a fever of 38.5°C. On admission, clinical examination revealed right upper quadrant tenderness and jaundice. Laboratory data (normal values in parentheses) showed 7.8 mg/dL total bilirubin (< 1.2 mg/dL), 986 IU/L alkaline phosphatase (< 40 IU/L), 519 IU/L γ -glutamyl transpeptidase (< 40 IU/L), 189 IU/L ALT (< 40 IU/L), 167 IU/L AST (< 40 IU/L), 484 mg/dL cholesterol, 878 mg/dL

Abstract

A 76-year-old diabetic man underwent cholecystectomy for gangrenous calculous cholecystitis. His postoperative course was complicated by the development of *Candida albicans* esophagitis necessitating antifungal therapy, and total parenteral nutrition (TPN) for 15 d. Seven weeks after cholecystectomy, he presented with cholangitis. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated extrahepatic filling defects. Despite endoscopic extraction of a biliary cast, cholestasis remained unchanged. Oral administration of ursodeoxycholic acid (UDCA), 750 mg/d, resulted in normalization of liver function tests. We, therefore, propose for the first time, combined endoscopic plus UDCA treatment for the management of biliary cast syndrome.

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Key words: Biliary cast; Ursodeoxycholic acid; Endoscopic retrograde cholangiopancreatography

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Figure 1 ERCP image revealing the dilated biliary tract with a tubular filling defect in the common bile duct until the junction of right and left hepatic ducts.

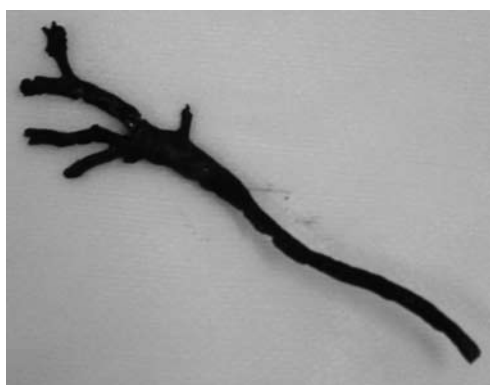


Figure 2 Biliary cast replicating the extrahepatic ductal system including the cystic duct stump.

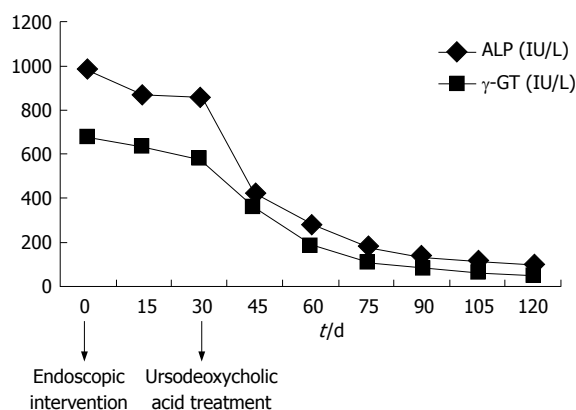


Figure 3 Effect of UDCA on cholestatic enzymes.

triglycerides and 18 000 cells/mm³ white blood cell count (< 10 000 cells/mm³).

Abdominal ultrasound and computed tomography demonstrated biliary tract dilatation without apparent lesion. At ERCP, a common bile duct tubular filling defect was found (Figure 1). Endoscopic sphincterotomy was performed, followed by basket retrieval of a cast of extrahepatic biliary tract (Figure 2). No residual cast in the visualized intrahepatic bile ducts was demonstrated. Cholangitis resolved, but cholestasis remained unchanged in the next month. UDCA (750 mg/d) was administered and a gradual improvement in cholestasis was observed with normalization of cholestatic enzymes after a 3-mo treatment period (Figure 3). After a 6-mo

treatment period, a repeated ERCP showed a normal biliary tract. The patient's condition was excellent with normal liver function tests 6 mo after cessation of UDCA treatment when we wrote this paper.

DISCUSSION

The biliary cast syndrome describes the presence of casts causing obstruction with its resultant sequelae of biliary infection, hepatocyte damage secondary to bile stasis and ductal damage, all contributing to cholangiopathy. The diagnosis is suspected in liver-transplant patients presenting with abnormal liver-associated enzymes of a cholestatic pattern, evidence of ductal dilatation on abdominal imaging studies or clinical evidence of obstruction, such as cholangitis or jaundice^[1-4] and is usually confirmed by ERCP.

To the best of our knowledge, there are only three reports of a total biliary cast in the non-transplant situation. D'Haens *et al*^[5] described an elderly lady with extensive cast formation that developed after initial recovery from emergency cholecystectomy. Despite multiple attempts at endoscopic removal, post-mortem findings confirmed the presence of diffuse bile casts and acute suppurative cholangitis. The authors suggest that, in the absence of a gallbladder, the increased bile pigment load may have initiated biliary cast formation. In the second report, Byrne *et al*^[6] described a patient with a history of traumatic head injury requiring admission to the intensive care unit, who developed a biliary cast. Despite endoscopic and surgical attempts at cast removal from large bile ducts, liver transplantation was ultimately performed for biliary cast formation. In a recent report, Gleeson *et al*^[7] described 2 patients with biliary cast syndrome in a non liver transplant setting. In the first case, biliary cast syndrome developed in a patient with no preexisting liver pathology. But, probably hepatic infarction induced by antiphospholipid antibody syndrome triggered the ischemic event leading to extrahepatic cast formation. In the second case, the etiology of cast formation was multifactorial, including fasting-related gallbladder hypocontractility, parenteral nutrition, biliary infection and possible ischemia due to hypotension-induced sepsis.

Biochemical analysis of biliary casts in liver-transplant patients showed that bilirubin seemed to be the main component (10%-50%) of casts and comprised conjugated free and oxidized varieties. Bile acids comprised 10%-15% of the cast sample and cholesterol represented about 5%-10%^[4]. The chemical composition of biliary casts in non-transplant patients is unknown due to its rarity. However, in both reported non-transplant patients^[5,6] and in our case, the biliary cast developed after difficult abdominal surgery in two patients, and severe head injury in one patient. They all had a long postoperative course with TPN. Our patient had similar characteristics (fasting, parenteral nutrition, biliary infection) with the second patient in Gleeson *et al*'s report^[7], while the underlying mechanism of biliary cast syndrome in the first patient was hepatic ischemia due

to antiphospholipid antibody syndrome. The similarities and differences of all reported non transplant patients with biliary cast syndrome underscore its multifactorial pathogenesis. It is known that sick patients, particularly abdominal surgical and neurosurgical patients, in intensive care units have been shown to develop biliary sludge frequently and rapidly^[8,9]. Fasting promotes gallbladder hypocontractility due to insufficient secretion of cholecystokinin and may, in part, explain the abdominal surgical patient group preference^[8,9]. Other risk factors for sludge formation include TPN, pregnancy and acquired immunodeficiency syndrome^[10,11].

The persistent cholestasis of our patient, despite removal of the extrahepatic biliary cast, is explained by the fact that development of cast was extensive and included the small intrahepatic ducts resulting in obstructive cholangiopathy. It is known that prolonged obstruction of the intra-and/or extrahepatic biliary tree due to stones or sludge, predisposes to caliber irregularities of the biliary system and also toxic effects of bile on hepatocytes with cholangitis further to contribute to cholestatic liver damage^[10].

We suppose that the mechanism by which the UDCA resolved our patient's cholestasis is the dissolution of biliary cast of small intrahepatic bile ducts because its main component is cholesterol. The presence of diabetes associated with a high level of cholesterol and combined with long-postoperative TPN contributes to hypersaturation of bile with cholesterol, and initiates biliary cast formation. Stone or sludge dissolution by UDCA is a complex physiochemical process, affecting mainly the cholesterol and phospholipid ionophase and enhancing cholesterol solubilization^[12,13]. It would be interesting if chromatography had been performed to reveal the components of our patient's biliary cast and confirm our hypothesis.

The beneficial effect of UDCA on biliary cast-associated cholestatic liver damage may be related to its hepatocytoprotective action, improvement in biliary acid secretion and immunomodulatory properties which reduce immune-related liver damage^[14,15]. Mechanisms underlying the beneficial therapeutic effects of UDCA on cholestatic liver diseases include: (1) protection of injured cholangiocytes against toxic effects of bile acids, (2) stimulation of impaired biliary secretion, (3) stimulation of detoxification of hydrophobic bile acids, and (4) inhibition of apoptosis of hepatocytes. However, it is not clear which of these mechanisms by which UDCA exerts its beneficial effects on cholestatic liver diseases. Most likely, such effects depend on the specific cholestatic liver disease and its stage^[16]. Knowing that biliary infection, bile duct damage and ischemia, fasting, hemolysis and abdominal surgery are all factors that may be implicated in biliary cast syndrome^[17], it would be interesting to use UDCA as a prophylactic in patients at risk of developing biliary cast syndrome. However, the

rarity of this complication makes such a study practically impossible.

In conclusion, administration of UDCA, if cholestasis remains unchanged after endoscopic removal of extrahepatic biliary cast, is a very promising treatment modality for biliary cast syndrome.

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LETTERS TO THE EDITOR

Management of constipation in the elderly: Emerging therapeutic strategies

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Abstract

A number of new, novel strategies for managing constipation in the elderly have emerged over the past few years. Prucalopride is one such new agent that is highly efficacious in managing chronic constipation. In fact, Camilleri *et al* in a recent study reported that the average number of bowel movements increased by at least one in nearly 47% of the patients who were administered a dose of 4 mg. Lubiprostone is another new agent recently approved by the FDA that shows efficacy in managing the symptoms of constipation. Neostigmine has also been successfully used for the management of recalcitrant constipation. Most of these studies have used subcutaneous neostigmine. Symbiotic yogurt containing components, such as Bifidobacterium and fructoligosaccharide, have also been recently shown to be highly effective in improving symptoms of constipation. Elderly patients especially those in hospices and nursing homes are often on opioids for pain management. Constipation secondary to opioid use is extremely common in nursing homes. Subcutaneous methylnaltrexone has recently been shown to be highly effective in the management of opioid-related constipation, and was recently approved by the FDA. Sacral nerve stimulation is another emerging strategy. A recent analysis by Mowatt *et al* supports the efficacy of this technique. Botulinum toxin is another agent that has already been successfully used for the management of chronic, refractory constipation in children and may be very effective for elderly constipation. Further larger studies are needed to confirm the findings noted in these studies. Constipation is clearly a major issue in the elderly and these new, emerging strategies will hopefully improve the quality of life and relieve the symptoms of constipation in this population.

TO THE EDITOR

The recent article by McCrea *et al* about the pathophysiology of constipation was highly enlightening^[1]. Interestingly, a number of new, novel strategies for managing constipation in the elderly have emerged over the past few years.

Prucalopride is one such new agent that is highly efficacious in managing chronic constipation. In fact, Camilleri *et al* in a recent study reported that the average number of bowel movements increased by at least one in nearly 47% of the patients who were administered a dose of 4 mg^[2]. Lubiprostone is another new agent recently approved by the FDA that shows efficacy in managing the symptoms of constipation. Lubiprostone is most efficacious when administered in a daily dose of 16 mg^[3]. Neostigmine has also been successfully used for the management of recalcitrant constipation. Most of these studies have used subcutaneous neostigmine administered in cancer patients^[4]. Symbiotic yogurt containing components, such as Bifidobacterium and fructoligosaccharide, have also been recently shown to be highly effective in improving symptoms of constipation^[5].

Elderly patients especially those in hospices and nursing homes are often on opioids for pain management. Constipation secondary to opioid use is extremely common in nursing homes. Subcutaneous methylnaltrexone has recently been shown to be highly effective in the management of opioid related constipation and was recently approved by the FDA^[6,7]. Sacral nerve stimulation is another emerging strategy^[8]. A recent analysis by Mowatt *et al* supports the efficacy of this technique^[9]. Botulinum toxin is another agent that

has already been successfully used for the management of chronic, refractory constipation in children and may be effective in elderly constipation^[10].

Further larger studies are needed to confirm the findings noted in these studies. Constipation is clearly a major issue in the elderly, and these new, emerging strategies will hopefully improve the quality of life and relieve the symptoms of constipation in this population.

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
 January 24-25, Frankfurt, Germany
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008
 February 14-16, Paris, France
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
 8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
 3rd Congress of ECCO - the European Crohn's and Colitis Organisation
 Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
 Canadian Association of Gastroenterology
 E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
 British Society of Gastroenterology Annual Meeting
 E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
 Asian Pacific Association for the Study of the Liver
 18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
 Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
 OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
 E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
 SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
 9th World Congress of the International Hepato-Pancreato Biliary Association
 Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
 43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008

May 21-22, California, USA
 ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
 E-mail: education@sege.org

June 4-7, Helsinki, Finland
 The 39th Nordic Meeting of Gastroenterology
www.congex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
 Semana de las Enfermedades Digestivas
 E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
 E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
 ESGAR 2008 19th Annual Meeting and Postgraduate Course
 E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
 16th International Congress of the European Association for Endoscopic Surgery
 E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
 E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
 10th World Congress on Gastrointestinal Cancer
 Imedex and ESMO
 E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)
 E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatologists.org

June 26-28, Bratislava, Slovakia
 5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
 ILTS 14th Annual International Congress
www.its.org

September 10-13, Budapest, Hungary
 11th World Congress of the International Society for Diseases of the Esophagus
 E-mail: isde@isde.net

September 13-16, New Delhi, India
 Asia Pacific Digestive Week
 E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE
 September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
 Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
 Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
 Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
 Third Annual Meeting
 European Society of Coloproctology
www.escp.eu.com



October 8-11, Istanbul, Turkey
 18th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists
 E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
 16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
 Anstralian Gastroenterology Week 2008
 E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
 71st Annual Colon and Rectal Surgery Conference
 E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
 59th AASLD Annual Meeting and Postgraduate Course
 The Liver Meeting
 Information: www.aasld.org

November 6-9, Lucerne, Switzerland
 Neurogastroenterology & Motility Joint International Meeting 2008
 E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
 1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
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N.O.T.E.S
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 Laparoscopic Digestive Surgery

June 27-28, November 7-8
 Laparoscopic Colorectal Surgery

July 3-5
 Interventional GI Endoscopy Techniques
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International Gastroenterological Congresses 2009
 March 23-26, Glasgow, Scotland
 Meeting of the British Society of Gastroenterology (BSG)
 E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
 Digestive Disease Week 2009

November 21-25, London, UK
 Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org



Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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Acknowledgments

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Format

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

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

Organization as author

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

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]

No author given

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

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

No volume or issue

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

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

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

Conference proceedings

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

Conference paper

- 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

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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