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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007

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EDITORIAL

What's new in inflammatory bowel disease in 2008?

Daniel C Baumgart

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Abstract

Ulcerative colitis and Crohn's disease represent the two major forms of inflammatory bowel disease. In this highlight topic series of articles we cover the latest developments in genetics and epidemiology, intestinal physiology, mucosal immunology, mechanisms of epithelial cell injury and restitution, current medical therapy, modern surgical management, important extra-intestinal complications such as primary sclerosing cholangitis, cholangiocellular carcinoma and autoimmune hepatitis as well as endoscopic and molecular screening, detection and prevention of small bowel and colorectal cancer.

Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Immunology; Genetics; Epidemiology; Medical therapy; Surgery; Cancer; Extra-intestinal manifestations

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In this special issue of the *World Journal of Gastroenterology* we have put together a group of expert faculty from all over the world to cover inflammatory bowel disease as a highlight topic.

Ulcerative colitis and Crohn's disease represent the two major forms of inflammatory bowel disease. Crohn's disease was first observed by the German surgeon Wilhelm Fabry (aka Guilhelmus Fabricius Hildanus) in 1623, and was later described by and named after the New York physician Dr Burril B Crohn[1,2]. Ulcerative colitis (UC) was first described by the London physician Sir Samuel Wilks in 1859[3].

Much has been learned since these early days about the etiology and particularly the genetic predisposition for these two disorders. The first paper in our series reviews the current knowledge about the etiology of inflammatory bowel disease. While all genome wide scans in the past have identified increased susceptibility genes, the most recently discovered susceptibility allele (IL23R) protects from Crohn's disease. The first article in our series puts this discovery in genetics in perspective with disease mechanisms and other etiological factors and its potential impact on the development of novel therapeutic strategies[4].

The most widely accepted hypothesis for the cause of inflammatory bowel disease is a disturbed interaction of the host immune system with the commensal microflora and other luminal antigens. This interaction begins at the epithelial layer and extends below to a multitude of other immune cells of the mucosal interface. Ultimately, mature and activated antigen presenting cells, (i.e. dendritic cells) induce and probably perpetuate an imbalance of effector and regulatory T-cell. Crohn's disease is a mainly a Th1 and Th17 mediated process, while ulcerative colitis appears to be predominately mediated through Th2 and NK T-cells. Two papers review the effects of these components in the immunopathogenesis of IBD[5,6].

Once the inflammation has occurred and is perpetuated by the dysfunctional immune system, the clinician is faced with distinct clinical phenotypes. Fortunately, the integrity of the gastrointestinal surface epithelium is rapidly reestablished even after extensive destruction. Rapid resealing of the epithelial barrier following injury is accomplished by a process termed epithelial restitution, followed by more delayed mechanisms of epithelial wound healing including increased epithelial cell proliferation and epithelial cell differentiation. Restitution of the intestinal surface epithelium is modulated by a range of highly divergent factors among them a broad spectrum of structurally distinct regulatory peptides, variously described as growth factors or cytokines[7].

Recent advances in our understanding of the pathophysiology of inflammation and in bioengineering have led to new therapeutic concepts targeting almost every aspect of the inflammatory process and help with the restitution of mucosal integrity. This provides clinicians with the opportunity of selecting from and combining conventional compounds and concepts as well as modern biologics and novel approaches to regimens tailored to the individual patient's needs[8].

Special issues in pediatric inflammatory bowel discussed are discussed in a separate paper[9].

Surgery has become an integral part of the management of inflammatory bowel disease and should not be considered a failure of medical management. Modern surgical techniques including minimally invasive procedures and current issues of surgical management are reviewed[10].

Inflammatory bowel disease is a systemic illness, not limited to the gastrointestinal tract. A substantial fraction of patients develops extra-intestinal manifestations, which itself can cause additional morbidity and complications. Hepatic manifestations and especially primary sclerosing cholangitis are among the most common, sometimes overlap and cause diagnostic and therapeutic challenges[11].

Finally, patients with chronic inflammatory conditions, including inflammatory bowel disease are prone to develop malignant complications. The final paper reviews current knowledge about the incidence and prevention of cancer[12].

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Primary sclerosing cholangitis, autoimmune hepatitis and overlap syndromes in inflammatory bowel disease

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Abstract

Primary sclerosing cholangitis (PSC) is a chronic progressive disorder of unknown aetiology characterised by chronic inflammation and stricture formation of the biliary tree. Symptoms include itch and lethargy and in advanced cases cholangitis and end-stage liver disease, however increasing numbers of asymptomatic individuals are being identified. The disease is rare in the general population but is strongly associated with inflammatory bowel disease (IBD) affecting up to 5% of patients with Ulcerative Colitis, with a slightly lower prevalence (up to 3.6%) in Crohn's disease. The strength of this association means that the vast majority (> 90%) of patients with PSC also have IBD, although many may have only mild gastro-intestinal symptoms. Usually IBD presents before PSC, although vice-versa can occur and the onset of both conditions can be separated in some cases by many years. Mean age of diagnosis of PSC is in the fifth decade of life with a strong male predominance. Risk is increased in those with a family history of PSC, suggesting a genetic predisposition and the disease is almost exclusive to non-smokers. The ulcerative colitis associated with PSC is characteristically mild, runs a quiescent course, is associated with rectal sparing, more severe right sided disease, backwash ileitis and has a high risk of pouchitis post-colectomy. Most worrisome is the high risk of colorectal malignancy which necessitates routine colonoscopic surveillance. Cholangiocarcinoma is also a frequent complication of PSC with a 10%-15% lifetime risk of developing this condition. Treatment with high dose ursodeoxycholic acid offers some chemoprotective effects against colorectal malignancy and may decrease symptoms, biochemical and histological progression of liver disease. Small duct PSC patients characteristically have normal cholangiography, and liver biopsy is required for diagnosis, it appears to have a more favourable prognosis. Autoimmune Hepatitis (AIH) is also more prevalent in patients with IBD, with up to 16% of patients with AIH also having ulcerative colitis. A small subgroup of patients have a AIH-PSC overlap syndrome and the management of these patients depends on liver histology, serum IgM levels, autoantibodies, degree of biochemical cholestasis and cholangiography as some of these patients may respond to immunosupression.

Key words: Primary sclerosing cholangitis; Autoimmune hepatitis; Liver disease; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

The first association between colonic ulceration and liver disease was made in 1874 by Thomas CH, who described a young man who died of a 'much enlarged, fatty liver in the presence of ulceration of the colon'[1]. The association was confirmed by James Lister in 1899, who reported a patient with ulcerative colitis and secondary diffuse hepatitis[2]. Over the next 100 years it has become well established that there is a close relationship between inflammatory bowel disease and various hepatobiliary disorders. These disorders are listed in Table 1.

PRIMARY SCLEROSING CHOLANGITIS

First described by Smith and Loe in 1965[3] Primary sclerosing cholangitis (PSC) is a chronic progressive disorder of unknown aetiology characterised by inflammation, fibrosis and stricture formation in medium and large sized ducts in the biliary tree[4,5]. Common symptoms include itch and lethargy although many patients (up to 45%) are asymptomatic, even with advanced disease[5]. The disease is strongly associated with Inflammatory Bowel Disease and may be detected as an incidental finding of a raised serum alkaline phosphatase. Magnetic resonance

Table 1 Hepatobiliary disorders associated with inflammatory bowel disease

Hepatobiliary disorders	Associated with	
	Ulcerative colitis	Crohn's disease
Primary sclerosing cholangitis (PSC)		
Large duct PSC	+	+
Small duct PSC ('pericholangitis')	+	+
Cirrhosis	+	+
Hepatoma	+	+
Cholangiocarcinoma	+	+
Miscellaneous disorders		
Fatty liver	+	+
Granulomas		+
Amyloidosis		+
Hepatic abscess		+
Gallstones		+
Autoimmune hepatitis	+	
Primary biliary cirrhosis	(+)	
Budd-Chiari syndrome	(+)	(+)

+: Definite association; (+): Possible association.

Table 2 Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis

Country of origin	Number of patients with ulcerative colitis	Percentage with primary sclerosing cholangitis
Oxford, UK[9]	681	2.9
Oslo, Norway[12]	336	4
Stockholm, Sweden[11]	1500	3.7 (5.5 in total colitis)

cholangography (MRCP) and endoscopic cholangiography (ERCP) are diagnostic, demonstrating the diffuse multifocal strictures and dilatation giving rise to a characteristic 'beaded' appearance (Figure 1).

Prevalence

PSC is rare in the general population. Data from Olmsted County (USA) in the year 2000 identified 20.9 cases of PSC per 100 000 of the population in men and 6.3 per 100 000 in women[6], which is similar to that seen in the UK[7]. There has been a suggestion that the incidence may be rising. In Spain the prevalence in 1988 was 2.24 per million having been 0.78 cases per million in 1984[8]. It is however unclear if this represents a true increase in the disease or improved case recognition due to improved diagnostics particularly in asymptomatic individuals.

PSC in IBD

Because of its strong association with inflammatory bowel disease (IBD) PSC is far more common in patients with ulcerative colitis (UC). The prevalence quoted in different case series varies widely, largely due to differences in the number of patients included with severe, active, or extensive inflammatory bowel disease studied and also in the method used to investigate liver dysfunction. Studies on unselected patients provide the most accurate data but are ethically difficult as both liver biopsy and ERCP are associated with significant morbidity. In an early study of unselected adult patients from Oxford, 5%-6% of the 300

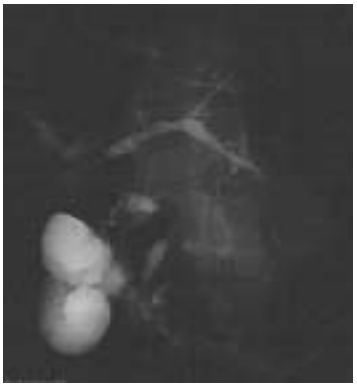

Figure 1 Endoscopic Retrograde Cholangiogram showing the classic 'beaded' appearance of bile ducts in Primary Sclerosing Cholangitis.

patients with ulcerative colitis had significant histological abnormalities on hepatic histology compared with 10% of 100 unselected patients with Crohn's disease; none of these patients underwent cholangiography[9,10]. Studies (Table 2) using selected patients include a large Swedish study of 1500 patients with UC where it was found that 5% of patients had increased serum Alkaline Phosphatase and, of those who underwent ERCP, 85% had evidence of PSC, being more common in men and those with pancolitis[11]. Other reports have found similar results. In a study from Oslo, 14% of patients had evidence of hepatobiliary pathology with 4% of patients with UC having PSC[12]. Thus it is generally accepted that approximately 5% of patients with UC will have associated PSC.

The percentage of Crohn's disease patients affected by PSC is much smaller[13]. In one study of 262 patients, 38 (15%) had abnormal liver function tests and underwent ERCP and liver biopsy, 9 of these (3.4% of the total) had PSC[14]. Crohn's disease associated with PSC is generally seen in patients who have extensive colonic or ileocolonic disease, or in paediatric populations.

IBD in PSC

Vice versa the great majority of patients with PSC have underlying IBD. The frequency with which it is recognised again varies widely depending on the intensity with which it is sought, varying from a minimum 25% of all PSC patients having IBD if identified by clinical history alone to 90% of patients if biopsies of rectum and sigmoid are taken at the time of flexible Sigmoidoscopy[15]. There are also large geographical variations in the prevalence of IBD in PSC (Table 3). Since the recognition of inflammatory bowel disease has important implications in terms of risk of colorectal cancer (PSC patients without IBD are not at increased risk of colorectal cancer and do not require colonoscopic surveillance[16]) it is the authors practice to perform colonoscopy and serial biopsies in all patients with newly diagnosed PSC.

Aetiology

The aetiology of PSC itself is unknown. The 2:1 male to female gender ratio of patients and the relatively poor response of the disease to immunosuppression suggest that PSC is not a classic autoimmune disease. However the association of PSC with IBD, autoimmune diseases, and a host of other humoral and cellular immune abnormalities

Table 3 Prevalence of inflammatory bowel disease in patients with primary sclerosing cholangitis (PSC)

Institution (country of origin)	Number of patients with PSC	Percentage with IBD
Royal Free Hospital (UK)[17]	29	72
Mayo Clinic (USA)[18]	50	70
Huddinge (Sweden)	305	72
King's College London (UK-children)	13	77
Okolicsanyi (Italy)[19]	82	54
Escorsell (Spain)	43	47
Okada (Japan)[20]	155	23

(Table 4) make an immunopathogenic mechanism likely. Particularly common serological abnormalities are raised IgM levels in up to 50% and positive anti-smooth muscle antibodies and antinuclear antibodies in up to 75%[21]. Anti Neutrophil Cytoplasmic Antibodies with a pericellular pattern (pANCA) are also a common finding, being present in 80% of patients being and unrelated to the presence or absence of UC. Cellular immunity anomalies include portal tract infiltration of functional T cells, restricted T cell receptor repertoire, and aberrant expression of HLA and costimulatory molecules on biliary epithelial cells.

Immunogenetic studies have identified a number of key HLA haplotypes (Table 5) associated with PSC, HLA-B8/DR3 haplotype being particularly common in patients with PSC and UC and infrequent in patients with PSC alone[22]. This haplotype is also associated with other organ-specific autoimmune disease including coeliac disease, thyrotoxicosis, lupoid autoimmune hepatitis, myasthenia gravis, and type 1 diabetes mellitus. However PSC, independent of HLA type, is also associated with a range of autoimmune diseases, diabetes mellitus and Graves disease being the most common. Saarinen *et al* found that 25% of patients with PSC had one or more autoimmune disease, compared to 9% of patients with inflammatory bowel disease alone[23].

Possible explanations for the PSC association with IBD include the development of autoantibodies to an unknown antigen in an immunogenetically susceptible host which cross reacts with biliary and colonic epithelium and is capable of inducing complement activation[24]. Alternatively, the initiation of the immune response may be the ingress of bacteria or other toxic metabolites through the diseased bowel wall.

CLINICAL COURSE

Onset

PSC is a disease of middle age with a mean age of diagnosis is 40 years in men and 45 years in women. Bowel symptoms have usually developed before those of PSC and UC is therefore usually diagnosed several years before PSC. However occassionally symptoms of PSC may precede IBD by up to 4 years[25]. The age of onset of the symptoms also has important implications as there is evidence to suggest patients with Colitis in childhood have a high prevalence

Table 4 Evidence for the influence of immune mechanisms on the aetiology of PSC

Humoral immunity	Increased circulating immune complexes
	Elevated immunoglobulin levels (IgG and IgM)
	Low titres of non-organ specific autoantibodies (ANA and SMA)
	High titres of antineutrophil antibodies
	Autoantibodies to BEC surface antigens
Cell mediated immunity	Decreased levels of circulating peripheral CD8+ve Tcells
	Portal T cell and NK cell infiltrate
	Increased activated and memory T cells
	Restricted T cell receptor repertoire (Vβ3)
	Aberrant expression of HLA-DR on BEC
	Coexpression of costimulatory molecules and HLA-DR on BECs
	Increased circulating and tissue bound adhesion molecules
Immune effector mechanisms	Enhanced cytokine expression in the liver
Immunogenetic mechanisms	HLA associations

Table 5 Key HLA haplotypes associated with primary sclerosing cholangitis

Haplotype	Significance in PSC
B8-TNF*2-DRB3*0101-DRB1*0301-DQA1*0501-DQB1*0201	Strong association with disease susceptibility
DRB3*0101-DRB1*1301-DQA1*0103-DQB1*0603	Strong association with disease susceptibility
DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602	Weak association with disease susceptibility
DRB4*0103-DRB1*0401-DQA1*03-DQB1*0302	Strong association with protection against disease
MICA*008	Strong association with disease susceptibility

of liver abnormalities than adults, with up to 60% children having abnormal liver function tests[26].

Risk factors

Unlike other immune mediated diseases PSC is far more common in men with 70% of all PSC patients being male. This male preponderance is absent in PSC patients without associated IBD, being equally common in both males and females[27]. In addition to IBD and male sex, another recognised risk factor is a family history of the disease, There is a 0.7% risk in first degree relatives of patients with PSC rising to 1.5% in siblings of affected individuals[28].

Smoking is a well recognized protective factor against the development of ulcerative colitis, and three studies have suggested that cigarette smoking may also additionally protect against the development of PSC. Moreover, this protective effect was more marked in patients with primary sclerosing cholangitis than ulcerative colitis and was seen in patients with and without inflammatory bowel disease[29-31]. The mechanism of protection in both disorders remains unknown.

Clinical characteristics of IBD associated with PSC

There are a number of unique features of Ulcerative

Colitis associated with PSC (Table 6). The colitis is often mild, asymptomatic and runs a quiescent course[32]. It is associated with rectal sparing[33]; and therefore not seen on rigid sigmoidoscopy; is often more severe proximally and associated with backwash ileitis. It may be visible microscopically with a relatively normal macroscopic appearance and pouchitis is far more common after colectomy and ileo-anal pouch formation, occurring in up to 60% of PSC patients compared to 15% in patients with UC alone[34].

Most importantly PSC is a significant risk factor for the development of carcinoma of the colon in patients with inflammatory bowel disease[35-37]. A meta-analysis demonstrated a four fold increase in colon cancer in UC patients with PSC when compared to UC alone (RR 4.26 95% CI 2.8-6.48)[38]. The risk of proximal cancer is particularly increased[37,39]. Additional risk factors are duration and extent of disease and a family history of colorectal cancer[40]. Current U.K. guidelines therefore recommend annual screening colonoscopy in IBD patients with PSC[41].

Treatment of PSC with ursodeoxycholic acid appears to have a chemopreventative effect decreasing the incidence of carcinoma of the colon and is therefore recommended[42]. Treatment with 5-aminosalicylic acid (5-ASA) compounds have also been shown to reduce the risk of colorectal cancer in general patients with UC[43]. There are no studies specific for PSC/IBD patients in this context but it is reasonable to infer that it will be protective and is also therefore recommended.

Prognosis

Despite treatment however PSC is generally progressive and prognosis poor. Median survival without liver transplantation after diagnosis is approximately 12 years[44] with survival worse for those who are symptomatic at presentation[45].

The major cause of mortality is the occurrence of cholangiocarcinoma, which is significantly increased in patients with PSC who display a 10%-15% lifetime risk of developing the disease. This risk is higher in patients with associated inflammatory bowel disease than those with PSC alone, with an estimated annual incidence of 0.5%-1%[46-48]. Unfortunately there remains no validated screening tool for cholangiocarcinoma and therefore tumours often present at an advanced stage and have a poor prognosis. Other complications of PSC include osteoporosis, this is a frequent finding the mechanism of which remains unclear[49]. Bone density measurement should therefore be considered in these patients.

PSC treatment

Treatments which have been tried in an attempt to prevent progression of the liver disease to cirrhosis and to decrease the risk of complications especially malignancy include tacrolimus[50], methotrexate[51], corticoteroids[52], D-penillamine[53], etanercept[54], cyclosporin[55], azathioprine, 6-mercaptopurine[56] and pentoxifylline[57]. None of these agents have demonstrated any consistent benefit.

Ursodeoxycholic acid (UDCA) is the only drug which has been demonstrated to have a positive effect. It is also the most extensively studied[58] with some, but not all, studies showing improvements in biochemistry, symptoms and histology[59-64]. The most promising data is for long term UDCA used at high dose, 20-30 mg/kg per day (in divided doses), as this may increase clinical benefit and decrease histological progression of disease[65,66]. In view of its chemopreventative effect against colon cancer and its low incidence of side effects, high-dose UDCA is currently the best available treatment for PSC.

Table 6 Clinical, Endoscopic, and Histological findings that characterize IBD-PSC

Male predominance
Quiescent colitis
Pancolitis
Rectal sparing
Backwash ileitis
Pouchitis
Colorectal dysplasia/carcinoma
Different extracolonic manifestations

The concurrence of both conditions also requires that the impact of treatment of either disease on the other must be considered. Proctocolectomy appears to have no effect on liver biochemistry, histology or survival of patients with PSC and chronic UC[67]. However the natural history of UC following liver transplantation is variable[68-72]. One study showed approximately a third had improvement in colitis, a third remained unchanged and a third suffered increased activity of colonic disease[73]. Several series report increased rates of colon cancer and colectomy after liver transplantation[74-76]. It remains unclear if this is due to longstanding immunosuppressant use or confounding variables such as long duration of disease.

SMALL DUCT PSC

A variant of the disease called 'small duct PSC' is applied to the small percentage of patients with characteristic clinical findings but who have a normal cholangiogram. The absence of macroscopic abnormalities necessitates liver biopsy for the diagnosis of small-duct PSC. Characteristic features of PSC on liver biopsy are concentric rings of connective tissue around bile ducts, the so-called 'onion ring' appearance[77]. Other features which are less specific include enlargement of the portal triads, periportal inflammation with mononuclear and polymorphonuclear cells, oedema and bile duct proliferation. Extension into the hepatic parenchyma with bridging fibrosis and ultimately cirrhosis represents advanced disease.

Small duct PSC appears to have a more favourable prognosis with a median survival 29.5 years compared to 17 years for classical PSC[78-81], although occasionally patients with small duct PSC progress to large duct disease.

AUTOIMMUNE HEPATITIS AND OVERLAP SYNDROMES IN IBD

Patients with IBD are also at increased risk of other of

immune mediated liver disease including autoimmune hepatitis (AIH). In one study, UC was present in 16% of patients with AIH[82]. AIH and PSC may also occur within the same individual and it remains unclear if this represents the independent occurrence of both diseases, either sequentially or concurrently, the presence of a distinct overlap syndrome or different stages in the evolution of a single disease entity.

Difficulties with definitions and thus classification of these patients has led to widely varying estimates as to the frequency of the coexistence of the two conditions with studies ranging from 7.6% to 53.8%. of patients with PSC have features of AIH[83-89]. and conversely an abnormal cholangiogram being present in 42% of patients with AIH and UC. The association between AIH and PSC appears more common in the paediatric population.

Patients with PSC should be suspected of having associated AIH or AIH-PSC overlap if they have interface hepatitis on biopsy, high serum IgG levels, lower ALP and the presence of autoantibodies (ANA or SMA titre > 1:40). It is important to identify these patients as they maybe responsive to treatment with steroids[85].

Patients with AIH should be suspected of having associated PSC or AIH-PSC overlap[90] if they have pruritis, UC, bile duct abnormalities on histology, cholestatic liver biochemistry (ALP × 2 ULN) and abnormal cholangiography. These patients tend to be unresponsive to steroids[87,91-93].

CONCLUSION

From the original descriptions of liver disease associated with disease of the bowel over a century ago much progress has been made. The association of inflammatory bowel disease with PSC, AIH and overlap syndromes is now well established and much is known on the epidemiology and course of the conditions. Although there remain many more questions than answers, an increasingly developed concept of hepatobiliary disorder in inflammatory bowel disease has emerged. It is suggested that the major hepatobiliary diseases seen in association with both UC and Crohn's disease, namely PSC, cirrhosis, cholangiocarcinoma, and most cases of autoimmune hepatitis, represent different aspects of the same spectrum of hepatobiliary disease. However what is cause, what is consequence and what is coincidence remains to be seen.

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Inflammatory bowel disease: Genetic and epidemiologic considerations

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Abstract

Genome-wide association studies have firmly established that many genomic loci contribute to inflammatory bowel disease, especially in Crohn's disease. These studies have newly-established the importance of the interleukin 23 and autophagy pathways in disease pathogenesis. Future challenges include: (1) the establishment of precisely causal alleles, (2) definition of altered functional outcomes of associated and causal alleles and (3) integration of genetic findings with environmental factors.

Key words: Crohn's disease; Ulcerative colitis; Interleukin 23; Autophagy; Complex genetics

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INTRODUCTION

The fields of genetics and epidemiology share a common goal of identifying specific factors, present in some individuals, and absent in others, that contribute to disease causation. As with many diseases, in inflammatory bowel disease (IBD), carefully performed epidemiologic studies provide an ongoing basis for the rationale and design of genetic studies. Observations on the nature and magnitude of familial clustering of IBD cases provided the framework for the present genetic observations that Crohn's disease (CD) and ulcerative colitis (UC) share some susceptibility alleles (e.g. *IL23R*) whereas others are unique to one disease subtype or the other. This review summarizes present understanding of the genetics and epidemiology of IBD, and describes the encouraging early results from genome-wide association studies which demonstrate significant consistency between studies.

EPIDEMIOLOGIC OBSERVATIONS FORM THE BASIS FOR GENETIC STUDIES IN IBD

Familial risk in IBD

The observation that cases of IBD cluster within families suggested that both shared genetic and environmental factors could contribute to disease pathophysiology. That monozygotic (MZ) twin concordance is significantly higher than dizygotic (DZ) twin concordance for both CD (20%-50% *vs* 0%-7%, respectively) and UC (14%-19% *vs* 0%-5%, respectively) indicates that genetic factors definitively contribute to both disorders[1-4]. The observation that, even among MZ twins, disease concordance is significantly less than 100% demonstrates the significant role that environmental and developmental factors play in disease pathogenesis. Like most genetic disorders, inheritance in IBD does not follow a simple Mendelian pattern. This would indicate that no single gene mutation is sufficient and/or necessary to contribute to disease, indicating that IBD represents complex, multigenic genetic disorders. In complex genetic disorders, any single gene mutation/polymorphism will not confer a simple determinative effect of disease development (as is the case for Mendelian, single-gene disorders), but rather merely confers a statistically increased, but non-determinative susceptibility to developing disease. Approximately 5% to 20% of patients with IBD have a family history of IBD[5,6]. A family history of IBD is generally more prevalent among CD than UC patients[6]. The relative risk to siblings, λs, (sibling risk compared to general population risk) ranges from 30-40 for CD and 10-20 for UC[7,8]. Furthermore, the relative risk for UC to siblings of a CD proband was observed to be 3.9 and the cross-disease relative risk to siblings of a UC proband was observed to be 1.8, suggesting that CD and UC are genetically related[9].

Comparative prevalence of IBD

The prevalence of CD and UC is highest in North America, northern Europe, and the United Kingdom, with averages ranging from 100 to 200 cases per 100 000[10-13]. IBD is more common in European Americans compared with African Americans, and the lowest rates of IBD have been reported in Hispanics and Asians[7,14-17]. The

prevalence of CD based on inpatient and outpatient visits in a Southern California Kaiser HMO study was 43.6 per 100 000 for European Americans, 29.8 per 100 000 for African-Americans, 5.6 per 100 000 for Asians and 4.1 per 100 000 for Hispanics[18]. However, two different Kaiser HMO studies found similar rates of hospitalizations among African-American and European ancestry individuals[18,19]. In addition, a more recent study demonstrated a similar incidence of IBD in African-Americans and European Americans[20]. Within European ancestry populations, the rates of IBD are higher in persons of Jewish ancestry than other ethnic groups[14,21], and higher in Ashkenazi (Central and Eastern European) Jews than in Sephardic (Middle Eastern and Spanish ancestry) Jews[14,21].

The hygiene hypothesis

Generally, IBD incidence is lower in developing countries and higher in industrialized countries, such as in North America and Europe; as a country undergoes the transition to industrialization, the prevalence of UC rises first, followed by a rise in the prevalence of CD[10]. The hygiene hypothesis proposes that lack of exposure to select microbial agents in childhood causes IBD[22]. In support of this, in Manitoba, patients with CD are less likely to have lived on a farm and less likely to have drunk unpasteurized milk. Furthermore, CD patients were more likely to have used tap water rather than well water, and more likely to have had a pet[23]. Against this hypothesis, a separate study of pediatric IBD observed that owning a pet, day-care attendance, and "physician diagnosed infection" between 5 and 10 years of age was associated with increased CD risk, and regular use of a personal towel and lesser crowding in the home with decreased risk[24].

The measurable changes in disease incidence in various geographic regions highlights the contributory role of environmental factors in IBD pathogenesis. A leading culprit for such environmental contributions is a changing intestinal intraluminal microbial milieu. At birth, the intestinal lumen is largely sterile[25], with rapid bacterial colonization ensuing in the neonatal period. There is some evidence to suggest that fecal colonization patterns may reflect environmental cues in the perinatal period and thus may be manipulable[26,27] as a potential means of preventing disease in high risk individuals.

Tobacco and IBD

Within Western countries, smoking is the most well established environmental risk factor, increasing risk for CD by approximately two-fold[28] and non-smoking decreasing risk for UC two- to five-fold[14]. Being an ex-smoker increases the risk of developing UC two-fold[29]. Ex-smokers make up an increasing percentage of older patients diagnosed with UC, accounting for more than 35% of the attributable risk of late onset (> 45 years) UC and a large component of the second peak in diagnosis[30]. Interestingly, Bridger *et al* observed that in IBD pedigrees with UC/CD sibling pairs, smokers tend to develop CD and non-smokers UC (O.R. 10.5)[31]. A significant increase in smoking in younger patients with familial CD has been reported[30]. Smoking is also associated with early recurrence of CD following surgery[32]. Future genetic studies in IBD will explore whether specific genetic associations will be stratifiable based on tobacco status at the time of diagnosis. However, tobacco may exert many of its phenotypic effects through epigenetic (reversible, changes in gene regulation that occur without a change in DNA sequence or genotype) mechanisms, such as DNA methylation effects on transcriptional activation[33].

HUMAN GENETIC VARIATION AND THE HAPMAP PROJECT

The most abundant of the human genetic variants are single nucleotide polymorphisms (SNPs). A SNP is a DNA sequence variation occurring when a single nucleotide, A, T, C, or G, in the genome differs between individuals, or between homologous chromosomes within an individual. Three salient features of SNPs include, (1) their allele frequencies within a population of interest, (2) their correlation, or linkage disequilibrium, with SNPs in the immediate genomic vicinity, and (3) whether or not the SNP of interest results in a measurable phenotypic change in protein function and/or expression, that is, represents a functional polymorphism.

As a very general rule, SNPs with a minor allele frequency approaching 50%, are genetically more ancient, having had a longer time to increase in frequency, and are more likely to be observed in all racial groups. In contrast, uncommon SNPs having a minor allele frequency of less than 5% (that is 5 of 100 chromosomes tested from 50 individuals carry the minor allele) are more likely to be evolutionarily more recent, and are often observed uniquely in one racial group or another. A number of factors besides the evolutionary age of a SNP can affect its frequency within a population, notably whether or not the SNP confers a selective advantage or disadvantage with respect to reproductive fitness within a population. It may be speculated that functional SNPs that contribute to the development of chronic inflammatory disorders such as IBD may confer a selective advantage with respect to combating historically significant infectious pathogens.

It is estimated that there are 5 million SNPs common SNPs having a minor allele frequency greater than 10%[34]. In order to comprehensively sample all common variation throughout the genome, however, it is not required that millions of SNPs be directly tested, due to the high degree of correlation, or linkage disequilibrium that exists between SNPs throughout the human genome. The Human HapMap Project empirically defined the linkage disequilibrium patterns in European, African and Asian cohorts and provided the basis for the genome-wide association studies that increasingly being reported for various complex genetic disorders[35]. It is estimated that genotyping several hundred thousand SNPs in European ancestry cohorts will sample nearly 80% of the common human variation with an r^2 (measure of linkage disequilibrium) of greater than 0.8[36].

The costs of genotyping several hundred thousand SNPs continue to decrease, and therefore genotyping sufficiently large cohorts to identify IBD genes through genome-wide association studies is now feasible. Once

associations with SNPs are identified, the challenge remains to identify the functional polymorphisms that account for the statistical association. The precedent from Mendelian disorders would suggest that those amino acid variants which directly affect protein structure are likely to disproportionately contribute to disease susceptibility. Within amino acid polymorphisms, those variants in highly conserved (between species) regions or contained within key functional domains are more likely to have significant functional effects. Ultimately, however, proof of disease contribution requires demonstration of altered functional effects associated with the mutation of interest.

IBD GENETIC ASSOCIATIONS PRIOR TO THE ADVENT OF GENOME-WIDE ASSOCIATION STUDIES

Because the statistical effects observed in multigenic disorders are relatively modest, the hallmark for accepting genetic associations is replication in independent cohorts. However, interpretation of disease association studies has been complicated by the reporting of often conflicting studies. In such instances, meta-analyses of all reported studies can provide some insight, with the caveat that publication bias (e.g. negative studies may be more difficult to publish than positive ones) may occur.

Nod2 (CARD15) associations to ileal CD

The most well-replicated IBD genetic associations is the Nod2 gene association with ileal CD[37,38]. Nod2 was the first definitive risk factor for CD and one of the first genes identified for a common complex genetic disorder. It functions as an intracellular sensor for bacterial peptidoglycan[39], and can be activated by a minimal bioactive component, muramyl dipeptide (MDP)[40,41]. MDP is present in both gram positive and negative bacteria and activates NF-κB and MAP kinase pathways[42]. Three uncommon (minor allele frequency less than 5% in healthy controls) polymorphisms, Arg702Trp, Gly908Arg, and Leu1007fsinsC are each highly associated with CD. These findings have been extensively replicated by a number of subsequent studies. A meta-analysis of 39 studies showed an odds ratio for simple heterozygotes of 2.4 (confidence interval, C.I. 2.0-2.9), and for homozygous/compound heterozygous carriers of 17.1 (C.I. 10.7-27.2)[43]. Nod2 carriage has been specifically associated with ileal involvement, stricturing complications and a modestly earlier age of onset.

Each of the three CD polymorphisms are located within or near the leucine rich repeat, sensing domain of Nod2, and each results in a decreased capacity to activate NF-κB in response peptidoglycan or MDP stimulation[39,44-48]. The frameshift mutation, Leu1007fsinsC demonstrates a largely complete deficiency in the capacity to signal in response to MDP stimulation[45]. Therefore, these functional polymorphisms represent direct risk alleles for CD. Importantly, the Nod2 discovery provides specific support for the long-held hypothesis that CD results from a genetically dysregulated host immune response to luminal bacteria. These Nod2 mutations have not been observed in Japanese, Chinese and Koreans with IBD[49-51], and they are rare in African-American IBD[52].

Despite the high odds ratios associated with the Nod2 mutations, it is estimated that the disease penetrance, even for homozygous or compound heterozygous carriers is limited, suggesting that these Nod2 variants alone are insufficient to produce disease. As each of the three major mutations demonstrates decreased function in primary human cells[39,44-48], Nod2-deficient murine models provide an important model for human disease. The absence of intestinal inflammation in Nod2-deficient mice further highlights the insufficiency of this pathway alone to induce CD[42,53]. Since the discovery of the CD-association with Nod2, a number of studies have added to understanding of the Nod2 functional pathway.

Given the dysregulation in intestinal immune homeostasis characteristic of CD, it is logical to hypothesize that Nod2 is important for either impaired tolerance mechanisms critical in limiting excessive activation of the intestinal immune system and/or altered initial defenses against intestinal bacteria. There are a broad range of tolerance mechanisms operating in the intestine to regulate excessive immune responses in the context of its unique exposure to a continuous bacterial load. Regarding initial defenses, a defect in bacterial recognition and responses at the intestinal epithelial surface might result in alterations in the population of commensal organisms, and/or increased invasion, which in turn, could contribute to an increased propensity toward intestinal inflammation. Nod2 is highly expressed in Paneth cells of the small intestine[54,55] and Nod2 mutations are associated with ileal location of disease[56]. Studies in humans and mice have shown that normal Nod2 function is required for optimal defensin expression and therefore, impaired defensin expression and regulation may contribute to the increased CD susceptibility observed in Nod2 risk allele carriers[57-59].

IBD5 association on chromosome 5q31

Within the IBD5 linkage region, association of CD with a 250 kb region on chromosome 5q31 was reported[60]. This association has been subsequently replicated in a number of studies[61-64], for both CD and UC[62,65]. Candidate polymorphisms found on this haplotype within the organic cation transporter OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*) genes have been reported[66,67]. However, statistically equivalent evidence for association has been observed throughout the risk haplotype[68] encompassing several genes, highlighting the need for additional genetic and functional investigation in this region. Phenotypic correlates have been reported for perianal disease[61,69], colonic location[70], disease complications and progression[68,71], extensive disease in UC[72], as well as for decreased height and weight at diagnosis in pediatric cohorts[73]. In contrast, some studies have not observed clear genotype-phenotype correlations[74,75].

HLA associations

Replicated HLA class Ⅱ associations in IBD include HLA-DRB1*1502 (serological marker HLA-DR2)[76] association with UC, and HLA-DRB1*0103 association with UC and colonic CD[77,78]. HLA-DRB1*0103 is noteworthy in that

it is a risk factor for both UC and colonic CD, suggesting it may play a role in chronic inflammation of the colon independent of major IBD phenotype (i.e., CD or UC)[78]. As with IBD5, the DRB1*0103 and DRB1*1502 class Ⅱ variants are in strong linkage disequilibrium with SNPs on multiple immunologically active candidate genes. Present approaches have not been able to discern whether these other genes or the class Ⅱ genes are the true risk genes in the HLA locus.

Reported, but unconfirmed IBD associations

Associations which have been reported within linkage regions to IBD but have not been consistently replicated include an indel polymorphism in the promoter region of the *NF-κB1* gene on chromosome 4q[79-82], multiple polymorphisms in the *MDR1/ABCB1* gene on chromosome 7q[83-92], and polymorphisms in the *DLG5* gene on chromosome 10q[70,93-102]. (See section C.4.1.) Haplotypes in the terminal exons of the chromosome 7p Nod1 (*CARD4*) gene and a Nod1 (*CARD4*) indel polymorphism were associated in two different study populations[47], although other studies have not replicated evidence for association[103-105]. Finally, studies of candidate genes that are not located within IBD linkage regions have revealed weak associations between UC and a variable number of tandem repeats polymorphism in the *IL1RN* gene[106] and between IBD and the TLR4 gene[107-113]. In a large case-control study, association was observed in UC and CD for Ala1011Ser within the Myo IXb (*MYO9B*) gene[114] that had been previously been implicated in celiac disease[115]. If replicated, this would indicate shared susceptibility pathways between multiple types of intestinal inflammation.

GENOME-WIDE ASSOCIATION STUDIES IN IBD

Emerging technologies now provide for the direct testing of a large number of SNPs throughout the genome in genome-wide association studies. The multiple genome-wide association studies reported below vary with respect to the genotyping platform utilized and the population cohort tested. Despite these differences, however, the first glimpses of comparative results between the genome-wide association studies are demonstrating encouraging consistency of findings between studies. Taken together, the early results from these studies would suggest that significant advances in defining well-replicated gene associations in IBD, especially in European ancestry cohorts, will shortly ensue.

TNFSF15 association to CD

The first genome-wide association study in IBD involved testing nearly 80 000 SNPs in a Japanese CD cohort[116]. The most significant findings implicated SNPs and haplotypes within the TNFSF15 (TNF superfamily) gene. These results were subsequently confirmed in European IBD cohorts[117]. TNFSF15 is a Th-1 polarizing cytokine which has been reported to be increased in IBD mucosa[118]. In a separate Belgian CD cohort, however, no significant evidence for association was observed[119]. These differences could reflect differences in markers genotyped and/or different contributions of susceptibility genes between Asian and European IBD cohorts. Future studies in a variety of population cohorts will provide important comparative insight.

IL23R is associated with CD and UC

A genome-wide association study testing over 300 000 autosomal SNPs was recently reported[120]. Three SNPs had nearly two orders of magnitude greater significance compared to the next most significant markers. Two of the three markers, were in the known CD susceptibility gene, Nod2. The third marker, rs11209026 ($P = 5.05 \times 10^{-9}$, corrected $P = 1.56 \times 10^{-3}$), was a non-synonymous SNP (Arg381Gln) in the *IL23R* gene on chromosome 1p31. Replication of these findings was observed in an independent cohort of 883 nuclear families and observed significant association in CD and non-Jewish UC. Therefore, *IL23R* represents both a CD and a UC susceptibility gene. The less common glutamine allele of Arg381Gln has an allele frequency of 1.9% in non-Jewish CD and 7.0% in non-Jewish controls and therefore protects against IBD[120]. Other variants within *IL23R* are also associated with IBD, independent of the Arg381Gln association. Since the initial report, these findings have subsequently been replicated in Scottish pediatric IBD[121] and Belgian CD[119] cohorts, indicating a clear role for *IL23R* in IBD susceptibility.

Given the IL-23 pathway's role in activation and perpetuation of organ-specific inflammatory responses, the genetic findings suggest that targeting the IL-23 pathway may be a rational therapeutic approach. In this regard, anti-p40 administration, which blocks both IL-23 and IL-12 activities, has proved promising[122]. The contribution of the *IL23R* pathway to IBD will likely involve more than simple gain or loss of function *IL23R* variants and ongoing studies of this pathway may reveal new therapeutic options. Future studies should examine mechanisms of the strong protective effect of the Arg381Gln allele could potentially be exploited to define clinical outcomes. The genetic association and the pro-inflammatory role of IL-23 strongly prioritize this pathway as a therapeutic target in IBD.

The *IL23R* genetic association to IBD correlates precisely with recent immunologic advances in understanding of the IL-23 pathway. A number of murine models of colitis, including IL-10 deficiency, T cell mediated (CD45Rbhigh reconstituted in RAG deficiency) and non-T cell mediated (agonistic anti-CD40 RAG deficiency, H. hepaticus-induced) colitis demonstrated significant amelioration when crossed with p40 and p19-deficient mice. This demonstrates the requirement for an intact IL-23 pathway for a variety of intestinal inflammation pathways[123-126].

A complete understanding of the strongly protective effect of *IL23R* polymorphisms in IBD should not be solely restricted to pharmacologic approaches to mimic the Arg381Gln polymorphism. While it is logical to test anti-p19 antibodies in IBD[127], the underlying paradigm is one of an ongoing requirement for continued immune

suppression. However, it is unlikely that the approximately 14% of European ancestry individuals heterozygous for the glutamine allele harbor significant immunodeficiencies, and therefore these individuals provide a unique opportunity to better understand the lifelong, dynamic host-intestinal microbial interactions that are the key to IBD. We are born with a sterile intestine[25] and an immature immune system[128], with the rapid acquisition of intestinal flora evolving dynamically with early instruction signals to the host immune response. The significant susceptibility of neonates to infection may correlate with their incapacity to induce IL-12p35 with lipopolysaccharide stimulation[129], with IL-23p19 induction being largely intact[130]. This would suggest that functional IL-23 pathway polymorphisms may be particularly important in modulating neonatal development of intestinal tolerance and bacterial colonization.

ATG16L association to CD

In a genome-wide survey of nearly 20 000 nonsynonymous SNPs, an amino acid polymorphisms, Thr300Ala within the *ATG16L1* gene was found to be highly associated with CD. The *ATG16L1* protein is comprised of N-terminal APG16 domain consisting of coiled coils and eight C-terminal WD repeats. The Thr300Ala variant is located at the N-terminus of the WD-repeat domain in *ATG16L1*. The *ATG16L1* gene is part of the autophagosome pathway and has been implicated in the processing of intracellular bacteria. Of interest, no association was observed in UC and a statistical interaction was reported with the Nod2 (*CARD15*) CD associations. Since the initial report, this association has been confirmed in a separate, Belgian CD cohort[119]. In addition, in this cohort, no association was observed in UC. Taken together, the *ATG16L1* association represents a well-replicated CD-specific association.

Association of a gene desert on chromosome 5p13.1 which modulates the expression of the prostaglandin receptor EP4 (*PTGER4*)

In a genome-wide association study in a Belgian CD cohort, in addition to the *CARD15*, *IL23R* and *ATG16L1* associations, association was observed in a region on chromosome 5p13.1. The most significant association was observed in a gene desert region flanked by a number of potential candidate genes, including *CARD6*, complement factors C6, C7 and C9 and the prostaglandin receptor, EP4 (*PTGER4*). No association was observed in UC for markers in this region. *PTGER4* is a compelling candidate, in part because *PTGER4* deficient mice develop a more severe colitis with dextran sodium sulfate treatment[131]. In elegant analyses, the investigators compared SNP data in this genomic region with mRNA expression levels of flanking genes from the corresponding, individual lymphoblastoid cell lines. Throughout the region of association, a number of SNPs were significantly associated with mRNA expression levels of *PTGER4*, including at least one SNP demonstrating both CD association as well as correlation with *PTGER4* expression. However, the susceptibility allele at this marker corresponds with increased *PTGER4* expression, which is not consistent with the increased colitic susceptibility observed in *PTGER4*-deficient mice[131]. However, taken together, these findings strongly suggest that genetic variation in this region is associated with IBD and significantly regulates *PTGER4* expression.

Assessment of genome-wide association studies in IBD

It is anticipated that the most significant associations observed in various genome-wide association studies will be largely replicable between studies in comparable population cohorts. Genome-wide association studies provide a comprehensive, unbiased landscape of common variation contributing to disease. Three relative limitations to these studies should be noted, however. Apart from the highly significant, outlying associations such as those observed for the Nod2 and *IL23R* gene associations, many of the most biologically significant disease associations may confer less significant statistical associations, and be obscured by surrounding "noise" conferred by false positive associations. Study design approaches to dissect true disease associations from surrounding noise remains a major methodological challenge moving forward. A second limitation is that the genotyping platforms typically utilized for these studies are limited to testing common genetic variation, with minor allele frequencies of greater than 5%. In the presence of significant allelic heterogeneity for uncommon variants within a disease gene, genome-wide association approaches will be relatively insensitive. The Nod2 associations are observed in genome-wide surveys so clearly because all three of the uncommon CD variants by chance share a common haplotype background. Therefore, the identification and characterization of uncommon variants may largely be missed by a complete reliance of genome-wide association approaches. Finally, many of the early genome-wide genotyping platforms were specifically developed to efficiently test European ancestry cohorts, and may not as efficiently assay Asian or African populations. Particularly for African populations which have shorter regions of linkage disequilibrium, testing of larger numbers of genetic markers will be required.

From genetics to genetic epidemiology: defining risks in patient subsets to assist clinical practice

A more complete pathophysiologic genetic definition of IBD will require integration of clinical observations, genetic data, statistical analyses, and delineation of underlying biologic processes. Interrogating GWA data for gene-gene interactions analyses has been proposed as being more powerful than traditional, single locus analyses under a range of interactive models. However such analyses may be more powerful if prior biologic knowledge on signaling pathways, *in vivo* models of inflammation and integration of genetic insight is applied. The growing catalogue of functional polymorphisms contributing to distinct chronic inflammatory disorders, while not necessarily demonstrating disease association, may contribute to IBD pathophysiology as disease modifiers. Stratification of genetic associations by phenotype variation and/or use of covariates may benefit genetic understanding of IBD by reducing heterogeneity that can obscure association evidence. Because of the strength of the Nod2 association

in CD, and because ileal CD represents a large fraction of overall cases of CD, the Nod2 associations were easily observed in the "all CD" analyses, and even the "all IBD" analyses. However, as the Nod2 genotype risk represents one of the largest among complex disorders, it would be anticipated that many IBD association signals may require correct subsetting, of which disease location is most well-established. Integration of clinical and genotype data may provide the capacity to predict complications and disease course in a clinically useful way. While the integration of definitive IBD risk alleles would be the most straightforward factors to include in such predictive models, it is possible that many key genetic factors (particularly well-established functional polymorphisms from related disorders) may, by themselves, not be associated with the major phenotypes, but rather function solely as disease modifiers. For example, it is possible that certain key genetic factors (particularly well-established functional polymorphisms from related disorders) may, by themselves, not be associated with the major phenotypes but rather serve to modify disease expression. Detecting such relationships requires a conditional analysis restricted to those with IBD in which certain phenotypic characteristics are modeled as a function of the presence of one or more genetic variants. Such models will also provide a framework for generating predictions about clinical course among newly diagnosed cases-providing clinicians important information for use in treatment planning.

The identification of major single gene associations such as *IL23R* and Nod2 provides a framework around which risk models for multigenic disorders can be developed. A major research interest moving forward will be identifying gene-gene interactions that contribute to increased disease risk or define pathophysiological subsets within the disease. This will be explored through: (1) a broad-based, systematic search of genomic regions demonstrating the most significant evidence for association, (2) pathway analyses integrating specific knowledge regarding functional human polymorphisms relevant to the *IL23R* and Nod2 pathways, and (3) studying key mechanistic intermediates known to be particularly relevant to disease pathogenesis.

As functional genetic variants that predispose to IBD are identified, their effect on modifying disease phenotype and/or disease course will need to be examined. For example, the CARD15 mutations increase susceptibility to CD generally, affect disease location (increase susceptibility to ileal location), and modify disease behavior. Because of the challenges of longitudinal follow-up, prospective evaluation of IBD-associated mutations represents a major challenge. The efficient abstraction of critical research datapoints from active clinical practices in an accurate and reproducible manner will be required. Interpretation of disease course from multi-center studies given differences in practice patterns represents an additional interpretative challenge. However, such studies will provide the basis for improved translation of genetic discovery in IBD and will need to be designed *via* active communication and collaboration throughout the IBD research community.

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Epithelial restitution and wound healing in inflammatory bowel disease

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Abstract

Inflammatory bowel disease is characterized by a chronic inflammation of the intestinal mucosa. The mucosal epithelium of the alimentary tract constitutes a key element of the mucosal barrier to a broad spectrum of deleterious substances present within the intestinal lumen including bacterial microorganisms, various dietary factors, gastrointestinal secretory products and drugs. In addition, this mucosal barrier can be disturbed in the course of various intestinal disorders including inflammatory bowel diseases. Fortunately, the integrity of the gastrointestinal surface epithelium is rapidly reestablished even after extensive destruction. Rapid resealing of the epithelial barrier following injuries is accomplished by a process termed epithelial restitution, followed by more delayed mechanisms of epithelial wound healing including increased epithelial cell proliferation and epithelial cell differentiation. Restitution of the intestinal surface epithelium is modulated by a range of highly divergent factors among them a broad spectrum of structurally distinct regulatory peptides, variously described as growth factors or cytokines. Several regulatory peptide factors act from the basolateral site of the epithelial surface and enhance epithelial cell restitution through TGF-β-dependent pathways. In contrast, members of the trefoil factor family (TFF peptides) appear to stimulate epithelial restitution in conjunction with mucin glycoproteins through a TGF-β-independent mechanism from the apical site of the intestinal epithelium. In addition, a number of other peptide molecules like extracellular matrix factors and blood clotting factors and also non-peptide molecules including phospholipids, short-chain fatty acids (SCFA), adenine nucleotides, trace elements and pharmacological agents modulate intestinal epithelial repair mechanisms. Repeated damage and injury of the intestinal surface are key features of various intestinal disorders including inflammatory bowel diseases and require constant repair of the epithelium. Enhancement of intestinal repair mechanisms by regulatory peptides or other modulatory factors may provide future approaches for the treatment of diseases that are characterized by injuries of the epithelial surface.

Key words: Intestines; Wound healing; Inflammation; Restitution

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THE MUCOSAL DEFENSE SYSTEM

The surface of the digestive tract is covered by epithelial cells that constitute an efficient physical barrier between the dietary and enteric flora pathogens found in the intestinal lumen and the individuum, but also allows an exchange between nutrients and the systemic circulation[1]. The epithelial defense mechanism can be categorized into three key components: pre-epithelial, epithelial and post-epithelial, the latter is represented by the lamina propria[2]. The pre-epithelial mucus barrier is composed of mucin associated with other proteins and lipids and forms a continuous gel into which a bicarbonate-rich fluid is secreted, maintaining a neutralizing pH at the epithelial surface. Phosphatidylcholine is the predominant surface bioactive phospholipid found within the gastrointestinal tract[3]. Intestinal epithelial cells secrete mucins and glycocalyx, which contain membrane-anchored negatively charged mucin-like glycoproteins and hydrophobic phospholipids[4]. The tight adherence of mucin to the apical surfaces of epithelia is owed to the existence of the specific complex between mucin oligosaccharides and the mucin binding protein of the apical mucosal membrane[5]. The hydrophobic lining of the luminal surface has an important functional role. It prevents microorganisms

Table 1 Important modulators of intestinal epithelial cell function

Localization	Modulator
Gastrointestinal lumen	Dietary compounds
	Alimentary secretions from salivary glands, stomach, pancreas or intestinal glandular cells
	Secreted regulatory peptides
	Physiological and pathogenic intestinal microflora
	Non-peptides factors (e.g. phospholipids, polyamines, short chain fatty acids and other)
	Drugs
	Other
Epithelium	Regulatory peptides
	IEL
	Local cell-cell interactions
	Non-peptide factors (e.g. phospholipids, adenine nucleotides, polyamines and others)
	Other
Lamina propria and basal lamina	Regulatory peptides expressed by various constituents of the lamina propria
	Extracellular matrix factors
	Neurotransmitters
	Nerval interactions
	Various mediators that are transported *via* the blood stream
	Non-peptide factors (e.g. phospholipids, adenine nucleotides, polyamines and others)
	Other

from getting into contact with and to adhering to the plasma membrane. It furthermore protects the mucosal epithelium against chemical and mechanical injuries[6]. Epithelial cells provide the second line of the mucosal defense system. Whereas in the upper digestive tract this layer consists of a stratified epithelium, the stomach, small, and large bowel are surfaced with a simple epithelial layer sealed by tight junctions[7]. When intact, the uptake of antigens, macro- and microorganism through this layer is restricted by luminal cell-surface structures. The mucosal surface epithelial cells are rapidly proliferating with a complete turnover every 24 to 96 h[8]. The proliferative compartment of epithelial cells is localized in the crypt region and is segregated from a gradient of increasingly differentiated epithelial cells present along the vertical axis of the functional villus compartment[9,10].

INTESTINAL WOUND HEALING

Damage and impairment of the intestinal surface barrier are observed in the course of various diseases and may result in an increased penetration and absorption of toxic and immunogenic factors into the body leading to inflammation, uncontrolled immune response, and disequilibrium of the homeostasis of the host. Thus, rapid resealing of the epithelial surface barrier following injuries or physiological damage is essential to preserve the normal homeostasis. Observations over the past several years have demonstrated the ability of the intestinal tract to rapidly reestablish the continuity of the surface epithelium after extensive destruction[11-14]. The continuity of the epithelial surface is reestablished by at least three distinct mechanisms. First, epithelial cells adjacent to the injured surface migrate into the wound to cover the denuded area. Those epithelial cells that migrate into the wound defect dedifferentiate, form pseudopodia-like structures, reorganize their cytoskeleton, and redifferentiate after closure of the wound defect. This process has been termed epithelial restitution and does not require cell proliferation[15]. Intestinal epithelial restitution occurs within minutes to hours both *in vivo* and *in vitro*. Secondly, epithelial cell proliferation is necessary to replenish the decreased cell pool. Third, maturation and differentiation of undifferentiated epithelial cells is needed to maintain the numerous functional activities of the mucosal epithelium. The separation of intestinal epithelial wound healing in three distinct processes is rather artificial and simplified. These three wound-healing processes overlap and distinct processes may not be observed *in vivo* where these processes overlap. The preservation of this barrier following injuries is regulated by a broad spectrum of structurally distinct regulatory factors, including cytokines, growth factors, adhesion molecules, neuropeptides and phospholipids[16-19]. However, this artificial and simplified model provides a tool to better understand the physiology and pathophysiology of intestinal epithelial wound healing. Moreover, deeper lesions or penetrating injuries will require additional repair mechanisms that involve inflammatory processes and non-epithelial cell populations. Inflammatory processes especially may interfere with epithelial cell migration and proliferation and thus modulate intestinal epithelial healing.

IMPORTANT MODULATORS OF THE INTESTINAL EPITHELIAL CELL FUNCTION

The epithelial cell populations of the intestinal mucosa are modulated by a number of factors that are present within the lumen, the epithelium itself or the underlying lamina propria (Table 1). Although the full variety of regulatory factors that play a role in the control of intestinal epithelial and non-epithelial cell populations has not been fully defined yet, there is increasing appreciation of the diversity of these factors in general and the importance of several specific peptide and non-peptide factors produced or released within the intestine. The identification and characterization of numerous regulatory peptide and non-peptide factors has led to the recognition of a network of interrelated factors within the intestine (Figure 1). The constituents of this network generally possess multiple functional properties and exhibit pleiotropism in their cellular sources and targets. As a result, this network is highly redundant in several dimensions[20]. Regulatory peptides especially seem to play a key role in intestinal epithelial wound repair, as they are abundantly detectable in the intestinal lumen, intestinal epithelium and the underlying lamina propria. Various members of several distinct regulatory peptide families have been recognized to modulate a broad spectrum of intestinal epithelial cell functions including cell migration, proliferation and/or differentiation (Figure 2). As outlined above the latter epithelial cell functions are highly relevant for the modulation of intestinal epithelial wound repair.

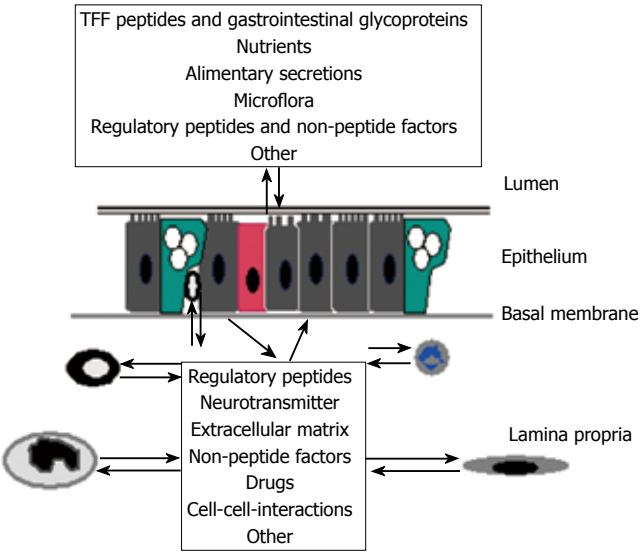

Figure 1 Regulatory network within the intestinal mucosa (adopted and modified from Reference 32).

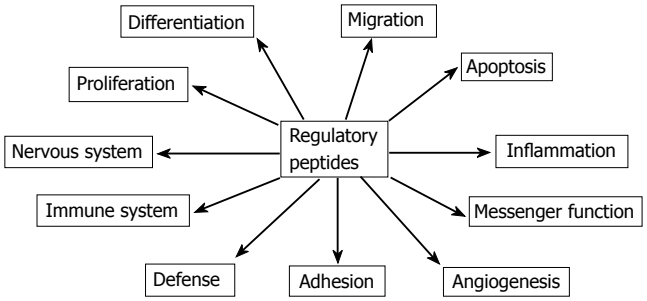

Figure 2 Functional activities of peptide growth factors within the intestinal mucosa (adopted and modified from Reference 32).

THE ROLE OF REGULATORY PEPTIDES FOR INTESTINAL EPITHELIAL WOUND HEALING

A broad spectrum of structurally distinct regulatory peptides is expressed from various cell populations within the mucosa of the intestinal tract. These regulatory peptides, conventionally designated as growth factors and cytokines play an essential role in regulating differential epithelial cell functions in order to preserve normal homeostasis and integrity of the intestinal mucosa[20-25]. The terminology of regulatory peptides is often confusing and arbitrary. The term cytokines is now increasingly used to describe a bunch of regulatory peptides that can be variously identified as regulatory peptides, peptide growth factors, interleukins, interferons and colony stimulating or hematopoetic stem cell factors. For the purpose of simplification, the term regulatory peptide or cytokine will be used in this paper to address all the different classes of regulatory peptide factors. Regulatory peptides can be reasonably classified on the basis of structural homologies and disparities into several discrete families. Peptide growth factor families and selected members with functional activities in the modulation of intestinal wound healing. In addition to growth factor families, a number of regulatory peptide factors, seemingly without structural similarities to other regulatory peptide family members like vascular endothelial cell growth factor (VEGF) and platelet-derived growth factor (PDGF) have been identified to be expressed within the intestinal tract and to modulate wound healing properties within the intestinal mucosa[25]. Furthermore, a countless number of classical cytokines like IL-1, IL-2, IL-15, IL-22 and IFN-γ are expressed within the intestine and modulate numerous intestinal epithelial cell functions[26-30].

The various effects of a number of regulatory peptides on cell adhesion, migration, proliferation, differentiation, intestinal epithelial barrier function and angiogenesis suggest that these peptides are likely relevant factors for intestinal repair mechanisms. Both *in-vitro* and *in-vivo* studies have demonstrated that several growth factors and cytokines can enhance epithelial cell restitution[31,32]. A spectrum of growth factors and cytokines including EGF, VEGF, HGF, GLP-2, various FGF peptides, IL-1, IL-2 and IFN-γ have been demonstrated to enhance epithelial cell restitution through a TGF-β-dependent pathway[33-36]. Interestingly, it appears that restitution-enhancing cytokines use different mechanisms to modulate TGF-β-peptide levels. While TGF-β, EGF, IL-1, IFN-γ and HGF only increased the concentration of bioactive TGF-β, acidic and basic FGF and also IL-2 enhanced both the bioactivation of TGF-β and the expression of TGF-β mRNA and production of latent TGF-β peptide. This might reflect a different mechanism, by which acidic and basic FGF and also IL-2 modulate the synthesis and bioactivation of TGF-β. In contrast to the above mentioned growth factors and cytokines that are assumed to act from the basolateral site of the epithelial surface and that seem to stimulate intestinal epithelial restitution through a common TGF-β-dependent pathway, various members of the trefoil factor family (TFF Peptide family) appear to stimulate epithelial restitution in conjunction with mucin glycoproteins through a TGF-β-independent mechanism from the apical pole of the epithelium[35,37,38]. In this regard, our group recently also demonstrated that mesalamine promotes intestinal wound healing *in vitro* through a TGF-β-independent mechanism[39]. Recent studies suggest that modulation of repair mechanisms by trefoil peptides may be mediated by modulation of the E-cadherin/catenin complex[40,41]. However, a double-blind, randomized, placebo-controlled study treating 16 patients with mild-to-moderate left sided ulcerative colitis with enemas containing human recombinant trefoil factor family-3 did not reveal any additional benefit above that of adding 5-aminosalicylic acid alone[42].

It has been demonstrated, that keratinocyte growth factor (KGF) has an important function in wound re-epithelialization[43] and KGF expression is strikingly increased in surgical specimens from patients suffering from Crohn's disease and ulcerative colitis[44]. Furthermore, in an experimental model of colitis, administration of KGF after but not before induction of colitis significantly ameliorated tissue damage demonstrating that exogenous KGF might promote IBD[45]. However, clinical studies

Table 2 Selected non-peptide factors with relevance for intestinal epithelial wound healing

Factor	Mechanism of action	References
Lysophosphatidic acid	Stimulates intestinal epithelial restitution and inhibits intestinal epithelial proliferation	[61,67]
Polyamines	Stimulate intestinal epithelial restitution and proliferation	[11,66,75]
Adenine nucleotides	Stimulate intestinal epithelial restitution and inhibit intestinal epithelial proliferation	[59]
Short chain fatty acids	Stimulate intestinal epithelial migration	[63,76]
Glutamine	Stimulates intestinal epithelial proliferation and migration	[76,77]

treating patients with CD or UC with exogenous KGF have not been performed yet. In addition to their potent effects on epithelial restitution, a number of regulatory peptide factors act also as potent modulators of epithelial cell proliferation[17,18,32,46-49]. The most important modulators of intestinal epithelial cell proliferation include EGF and TGF-β which both act as potent stimulators of intestinal epithelial proliferation and TGF-β which inhibits intestinal epithelial cell proliferation and plays an important counterbalancing role in the regulation of intestinal epithelial cell proliferation. TGF-β is the most potent inhibitor of intestinal epithelial cell proliferation overriding the stimulatory effects of other stimulatory factors. The growth stimulating effect of IL-2, FGF peptides, IGF and HGF is rather moderate compared to the effects of EGF and TGF-β that stimulate epithelial cell proliferation five- to ten-fold in several intestinal epithelial cell lines *in vitro*[33,34,48,50]. Thus, it is not astonishing that in a randomized, double-blind clinical trial, after a 2-wk treatment period, patients receiving EGF enemas had a significant lower disease activity score than the control patients[51]. Concerning basic FGF, in a mouse DSS-model of experimental colitis, rectal administration of human recombinant basic FGF ameliorated the inflammation score and suppressed TNF-α gene expression in the colonic tissue[52,53]. Recently, the important role of Glucagon-like-peptide-2 (GLP-2), which is secreted from local neuroendocrine epithelial cells and promotes epithelial cell proliferation *via* stimulation of enteric neurons[54], has received more attention. GLP-2 demonstrating ability to ameliorate murine short bowel syndrome and experimental colitis[55,56] has consequently lead to clinical studies, evaluating the effect of GLP-2 in patients with short bowel syndrome.

However, it has also to be considered that cytokines have pleiotropic activities. For example, FGF also induces stricture formation in Crohn's disease[57] and GLP-2 might have undesired effects in tumorogenesis[58], which might limit their therapeutic use. In addition, it has to be considered that these factors and also various non-peptide factors may act in an additive or even synergistic fashion which may potentiate their single effects. Notably, in addition to TGF-β the TGF-β family member Activin A has been identified to also inhibit epithelial cell proliferation, thus providing an additional mechanism to counterbalance the effects of proliferative factors present within the intestinal mucosa and to inhibit unrestrained cell growth[59,60].

MODULATION OF INTESTINAL EPITHELIAL WOUND HEALING BY NON-PEPTIDE FACTORS

In addition to the potent modulation of intestinal epithelial wound healing by regulatory peptides, it is increasingly appreciated that a broad spectrum of non-peptide factors exerts potent effects on intestinal epithelial cell populations and modulates those epithelial cell functions that are involved in the healing of intestinal injury (Table 2). These non-peptide factors encompass a broad spectrum of unrelated factors like phospholipids, nutrients (adenine) nucleotides, polyamines, short chain fatty acids (SCFA), products of the intestinal microflora, trace elements, pharmacological agents and other factors. Some of these factors are released by injured or dying mucosal cell populations (e.g. adenine nucleotides, phospholipids), other reach the intestinal mucosa *via* the intestinal lumen or the blood stream. These non peptide factors may exert growth factor like activities and exert potent effects on cell growth and differentiation in different cell populations including fibroblasts, vascular smooth muscle cells, endothelial cells and keratinocytes[20,35,61-63]. As some of these non-peptide factors are stable within the gastrointestinal tract despite high concentrations of acid, bile salts, proteases and microorganisms and as they exhibit only limited toxicity *in vivo*, they may serve as potential future targets to improve the armamentarium for the healing of mucosal epithelial injury.

Especially phospholipids and polyamines seem to be of special interest, as these non-peptide factors can be easily added to the regular diet and their overall content and biological activity can be modulated by various pharmacological agents[62,64-66]. Lysophosphatidic acid (LPA) is a key intermediate in the early steps of phospholipid biosynthesis and is rapidly produced and released from thrombin-activated platelets and growth factor stimulated fibroblasts to influence target cells by activating a specific 38-40 kDa G-protein coupled receptor that is expressed in many cells[67]. As a product of the blood-clotting process, LPA is a normal constituent of serum, where it is present in an albumin-bound form in physiologically relevant concentrations[64]. Major sources of LPA in the vicinity of injured epithelial cells are activated platelets, stimulated fibroblasts and presumably injured cells that release LPA due to non-specific phospholipase activation[67]. LPA promotes platelet aggregation and induces cellular tension and cell surface fibronectin assembly[68], which are also important events in wound repair suggesting an important role of LPA in inflammatory disorders. This was confirmed by our group when we demonstrated, that LPA not only promotes epithelial wound healing *in vitro* by a TGF-β-independent pathway, but also ameliorates experimental colitis in an experimental model of colitis in rats[61]. Interestingly, also lysophosphatidylethanolamine and lisofylline, which decreases lipid peroxidation, significantly

reduced the degree of inflammation and necrosis in an experimental colitis model[69], demonstrating that the administration of anti-inflammatory lysophospholipids and suppression of pro-inflammatory lipid metabolites by lisofylline may provide new approaches to ameliorate intestinal inflammation. This beneficial effect could also be demonstrated in other diseases since lisofylline and its analogs reversed autoimmune diabetes in a non-obese diabetic (NOD) mouse model and thus might act as a potential treatment for Type 1 diabetes[70,71].

Many studies have reported that bone marrow cells may have the potential to contribute to the repair of many non-hematopoetic tissues, including the intestinal epithelial cells. Bone-marrow derived cells are capable of promoting regeneration of damaged intestinal epithelial cells[72]. However, the underlying effects are not fully understood[18]. Nevertheless, bone-marrow transplantation combined with immune-suppressive therapy improves epithelial wound healing[73] and recombinant granulocyte-macrophage colony-stimulating factor have a therapeutic effect in patients with active Crohn's disease[74].

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TOPIC HIGHLIGHT

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Treatment of inflammatory bowel disease: A review of medical therapy

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory diseases of the gastrointestinal tract. While a cure remains elusive, both can be treated with medications that induce and maintain remission. With the recent advent of therapies that inhibit tumor necrosis factor (TNF) alpha the overlap in medical therapies for UC and CD has become greater. Although 5-ASA agents have been a mainstay in the treatment of both CD and UC, the data for their efficacy in patients with CD, particularly as maintenance therapy, are equivocal. Antibiotics may have a limited role in the treatment of colonic CD. Steroids continue to be the first choice to treat active disease not responsive to other more conservative therapy; non-systemic steroids such as oral and rectal budesonide for ileal and right-sided CD and distal UC respectively are also effective in mild-moderate disease. 6-mercaptopurine (6-MP) and its prodrug azathioprine are steroid-sparing immunomodulators effective in the maintenance of remission of both CD and UC, while methotrexate may be used in both induction and maintenance of CD. Infliximab and adalimumab are anti-TNF agents approved in the US and Europe for the treatment of Crohn's disease, and infliximab is also approved for the treatment of UC.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Medical therapy

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the principle syndromes encompassed by the classification of inflammatory bowel disease (IBD). While CD can affect any part of the gastrointestinal tract, it most commonly occurs in the distal ileum and colon, whereas UC by definition affects only the colon. The etiology appears multifactorial: an underlying immune dysregulation coupled with an intolerance to gut flora seems fundamental to the pathogenesis that, in some cases, are associated with genetic mutations or are initiated by environmental factors. Apart from a total proctocolectomy for UC, there is no cure for IBD. Medications, however, aid in the induction and maintenance of remission, and target various points along the disordered immune pathway implicated in IBD.

CROHN'S DISEASE

Aminosalicylates

While there is solid data supporting 5-aminosalicylic acid (5-ASA, mesalazine or mesalamine) in the induction and maintenance of remission for UC, their efficacy in the treatment of CD is not as clear. Interpretation of the data is often confounded by the use of different formulations, doses, and varied applications to different disease scenarios (disease location, concomitant medications, and prior therapies). 5-ASA agents are likely to have multiple anti-inflammatory effects, including inhibition of cyclooxygenase, lipoxygenase, B-cells, and several key inflammatory cytokines. Most recently, 5-ASA has been shown to activate selective peroxisome proliferators-activated receptor ligand-γ (PPAR-γ), a nuclear receptor that controls cell proliferation and apoptosis[1]. Originally designed as treatment for rheumatoid arthritis (RA), sulfasalazine was discovered to also benefit patients with IBD[2]. An azo-bond links sulfapyridine to 5-ASA and is cleaved by bacterial azo-reductase in the colon, thus allowing delivery of the active 5-ASA moiety to

the large intestine. Limitations of sulfasalazine include allergic reactions and side effects, largely attributed to the sulfapyridine moiety, as well as its lack of efficacy in isolated small bowel disease that is proximal to the colonic release of 5-ASA. Two newer non-sulfa-containing 5-ASA agents, balsalazide and olsalazine were developed to treat colonic inflammation; further, mesalamine formulated to release in a pH (Asacol®, Claversal®, Mesasal®, and Salofalk®) or time-dependent manner (Pentasa®) can treat either small or large bowel CD[2].

Active disease

The National Cooperative Crohn's Disease Study (NCCDS) and European Cooperative Crohn's Disease Study (ECCDS) were large multicenter randomized controlled trials published in 1979 and 1984 that evaluated the comparative efficacy of sulfasalazine, prednisone and azathioprine in the treatment of both active and quiescent CD. While the NCCDS found sulfasalazine 6 g/d superior to placebo overall in treating active disease, when stratified by disease location only those with colonic and ileocolonic (but not isolated small bowel) disease obtained benefit[3]. In contrast, the ECCDS did not demonstrate efficacy for sulfasalazine 3 g/d alone, but only in combination with 6-methylprednisolone[4].

Subsequently, newer mesalamine agents have been evaluated in clinical trials for CD. In the largest of these studies (n = 310), patients with active ileal or ileocolonic CD were randomized to receive Pentasa®, 1, 2 or 4 g/d or placebo. The 4 g/d group experienced a greater decrease in CDAI than the placebo group (72 *vs* 21 points, $P < 0.01$), an effect more pronounced in isolated ileal disease, and remission was achieved in 43% *vs* 18% respectively[5]. Subsequently, two similarly designed trials described in a meta-analysis failed to replicate these findings although there was an overall statistical benefit for the 4 g dose of mesalamine that was of questionable clinical significance[6,7]. Several other trials also have demonstrated benefit for mesalamine in CD, but the quality of the trials was less robust[8,9]. When compared to other agents in controlled trials approximately 40%-55% of patients treated with mesalamine 4 g/d achieve remissions but the efficacy was less than budesonide (9 mg/d) for the induction of remission at both 8 wk (45% *vs* 65%, $P = 0.001$) and 16 wk (36% *vs* 62%, $P < 0.001$)[10] and comparable to ciprofloxacin 1 g/d[11].

Maintenance after medical remission

Sulfasalazine at reduced doses compared to the induction phase provided no benefit compared to placebo in the maintenance phase of the NCCDS and ECCDS, nor in a smaller study[3,4,12]. Gisbert *et al* have reviewed nine randomized placebo-controlled studies of mesalamine as a maintenance agent, four of which showed a significantly decreased risk of relapse compared to placebo, although there was great heterogeneity in formulation, dosage, duration of treatment, and disease location[13]. Further, a Cochrane review of seven randomized placebo-controlled trials concluded that treatment with 5-ASA agents for at least six months did not confer an advantage over placebo in patients with medically-induced remission[14]. When initiated within three months of a medically-induced remission, mesalamine (2 g bid), in contrast to placebo, prevented more relapses over a two year period[15]. In the context of a steroid-induced remission, short-term weaning from steroids may be slightly facilitated with mesalamine 4 g/d, but there was no benefit at one year in relapse rate between patients maintained on mesalamine compared with placebo[16].

Post-operative maintenance

The natural history of CD after ileocolonic resection is variable, and may be influenced by such factors as pattern, extent, and duration of disease pre-operatively as well as smoking history. Endoscopic recurrence rates range from 28%-93% at one year[17], while clinical relapse rates have been reported at 20% and 34% at one and three years respectively[18]. Approximately 30% of patients require re-operation within 10 years[17], highlighting the relevance of identifying an effective post-operative maintenance strategy. Except for one study that showed a benefit at one (but not three) years, sulfasalazine has not been statistically superior to placebo in preventing post-operative relapse[17]. Data for mesalamine has been equivocal in the setting of post-operative maintenance trials. While a meta-analyses of 15 randomized controlled studies (n = 2097) of mesalamine as a maintenance medication in CD found a 13% pooled risk reduction for those patients with surgically-induced remissions[19], the largest (n = 318) and most rigorously conducted trial to date in which patients began mesalamine therapy (4 g/d) within ten days of surgery did not show benefit over placebo. While a post-hoc analysis did suggest efficacy for patients with isolated small bowel disease (21% *vs* 39% relapse rate, $P < 0.02$)[20], if this trial had been included in the meta-analysis, the overall findings of benefit compared to placebo would no longer have been significant[21]. Most recently, mesalamine at 3 g/d was inferior to mercaptopurine at 50 mg/d at preventing post-operative recurrence[22].

In summary, for the treatment of mild to moderate active CD, 5-ASA agents, while less efficacious than budesonide for ileal and or right colonic disease, may be a reasonable choice as first-line therapy: sulfasalazine should be reserved for patients with predominantly colonic disease, while time or pH-dependent release mesalamine are appropriate for patients with small bowel disease. The role of 5-ASA as a maintenance medication is equivocal at best, but is clearly of no benefit in patients with a steroid-induced remission and in the setting of post-operative maintenance, at least 3 g/d would need to be initiated immediately after surgery to provide any benefit for patients with small bowel disease.

ANTIBIOTICS

Active disease

Metronidazole, ciprofloxacin, combination anti-mycobacterials, and most recently ornidazole and rifaximin have been evaluated in the treatment of active CD. The few randomized controlled trials to study the efficacy of metronidazole and/or ciprofloxacin have been mostly small and provided negative results[7] despite subgroup

analyses suggesting a trend towards significant benefit in patients with colonic disease[23-25]. One small study (n = 47) showed ciprofloxacin 1 g/d for six months decreased CDAI scores significantly more than placebo ($P < 0.001$)[26] and while an eight month cross-over study between sulfasalazine 3 g/d and metronidazole 800 mg/d showed no treatment differences in the initial four months, 15 patients who switched from sulfasalazine to metronidazole had significant decreases in the CDAI compared to none of the group who crossed-over from metronidazole to sulfasalazine[27]. As previously described, 16-wk remission rates were similar for ciprofloxacin and mesalamine in a small, randomized trial[11] while another trial reported no differences in remission rates between the combination of ciprofloxacin and metronidazole *versus* methylprednisolone, despite a trend favoring steroids[28]. In contrast, a combination of ciprofloxacin and metronidazole provided no additional benefit over budesonide, alone, aside from a post-hoc analysis for patients with colonic disease[25]. A recent study that compared rifaximin 800 mg bid, 800 mg/placebo and placebo bid failed to show a significant difference between the three groups in clinical response or remission, despite a trend toward benefit with the higher dose[29].

Perianal disease and post-operative maintenance

Although antibiotic therapy is frequently used in the treatment of perianal fistulae, there are no randomized controlled trials to support this practice. Data from several small open-label trials conducted in the early 1980s reported the efficacy of metronidazole in healing perianal fistulae[30-32]. In the post-operative setting a three month course of metronidazole (20 mg/kg per day) decreased the severity of endoscopic lesions at one year (but not at two years) and delayed onset of clinical recurrence[33]. Most recently, ornidazole (1 g/d), started within 10 d of resection and continued for one year, showed significant benefit over placebo in both clinical and endoscopic recurrence rates[34]. The main limitation of long-term metronidazole and ornidazole is peripheral neuropathy.

In summary, while antibiotics are used frequently to treat perianal disease, their role in the treatment of active luminal disease and a safe and effective dose schedule in the post-operative setting, remain to be established.

SYSTEMIC STEROIDS

Mechanism of action

By binding to intracytoplasmic glucocorticoid receptors found in most cell types, glucocorticosteroids activate glucocorticoid-responsive elements (GREs), resulting in a broad spectrum of effects on the immune system including inhibition of the recruitment and proliferation of lymphocytes, monocytes and macrophages, migration of neutrophils to sites of inflammation, and decreased production of soluble inflammatory mediators including cytokines, leukotrienes, and prostaglandins[35].

Natural history

The natural history of 171 CD patients diagnosed between 1970 and 1993 has been studied in the Olmsted County, Minnesota population[36]. Of this cohort, only 43% ever required steroids before 1997 and of these, 58% were in complete remission after one month while 26% were in partial remission and 16% had no response. Of those who responded, the one-year outcomes were concerning as only 32% of patients had a prolonged response to corticosteroids, 28% became steroid-dependent and 38% had undergone surgery. These are data that are similar to the reported Danish, Copenhagen County experience[37]. Both exemplify the likelihood of developing steroid refractory or dependent disease with an accelerated course toward surgery. Hence, the requisite for steroids may be considered the "tipping point"[38] of CD that heralds a more complex subsequent course[39], including the need for surgery or the addition of an immunomodulatory agent.

Efficacy

Glucocorticosteroids are effective inductive agents for CD. The first definitive data came from the NCCDS, in which 60% of patients treated with prednisone (0.25-0.75 mg/kg per day) were in remission at 17 wk compared to 30% of placebo-treated patients[3]. Even more impressive were the results from the ECCDS in which 80% of patients treated with methylprednisolone (48 mg) achieved remission at 18 wk compared to less than 40% of placebo patients[4]. More recent randomized controlled studies have compared prednisolone (40 mg) or 6-methylprednisolone (48 mg) to budesonide (9 mg) in the treatment of active CD ileocolitis, with similar rates found for the induction of remission at 66% and 73% for the two systemic steroids[40,41].

Although in one retrospective review, 60% patients treated with alternate-day prednisone treatment (mean dose of 25 mg q.o.d.) maintained "favorable responses" for an average of 6.6 years[42], the overwhelming evidence does not support the use of corticosteroids for maintenance of remission. Neither the NCCDS nor ECCDS studies showed benefit of corticosteroids over placebo in maintaining remissions[3,4]. Conventional corticosteroids are not effective at preventing post-operative relapse[43] and a recent Cochrane review of three randomized double-blind placebo controlled studies showed no benefit of corticosteroid therapy in preventing relapses in patients with quiescent CD over 24 mo[44].

NON-SYSTEMIC STEROIDS

Budesonide, in delayed or controlled-release formulations that deliver the potent glucocorticoid to the ileum and/or right colon, has low systemic side effects owing to a high (80%-90%) first-pass metabolism[45]. Two randomized controlled studies demonstrated superiority of budesonide in the induction of remission in patients with ileal or ileo-right colonic disease[46]. In the first trial, 258 patients received 15, 9, or 3 mg of budesonide, daily, or placebo, with 43%, 51%, 33% and 20% of patients respectively achieving clinical remission in 8 wk ($P < 0.001$, $P = 0.009$ for the higher doses compared to placebo respectively)[47]. In the second study (n = 200), 9 mg/d, 4.5 mg BID twice daily budesonide or placebo yielded remission rates of 48%, 53%, and 33% respectively after 8 wk of treatment.

Although differences between the groups were not significant, when data from the two treatment groups were pooled, the budesonide group had a significantly greater decrease in CDAI than the placebo group ($P < 0.05$)[48]. One study comparing daily 18 mg, 9 mg, and 6 mg of budesonide found a dose-dependent effect, with 66%, 55% and 36% achieving remission. While for most patients, 9 mg/d is a sufficient dose, high disease activity (CDAI $\geqslant$ 300) or disease distal to the transverse colon responded better to the highest budesonide dose[49] and as discussed above, budesonide 9 mg/d has also been shown to be a more effective treatment than mesalamine for the induction of remission in mild-moderate active ileal and right-sided colonic CD[10]. When compared to prednisone, budesonide 9 mg/d there were no significant differences found in clinical remission rates[40,41,50,51] although a meta-analysis revealed the pooled rate difference of response of budesonide *vs* conventional corticosteroids to be - 8.5%, $P = 0.02$[52]. Budesonide was associated with fewer steroid side effects overall in three studies[40,41,50] and reduced incidence of moon facies and adrenal impairment in the other[51].

While extended treatment with budesonide has been shown to prolong the time to relapse compared to placebo, the difference was not sustained at one year with 3 mg[53] or 6 mg[54-56]. Similarly, another study found no difference in relapse rate at any time point over a one year period between patients treated with either 3 mg or 6 mg budesonide and placebo[57]. Neither budesonide 3 nor 6 mg/d was shown to be more effective than placebo in preventing post-operative clinical[58] or endoscopic recurrence[58,59]. Both a Cochrane review and meta-analysis confirmed that budesonide is ineffective at maintaining CD remissions[52,60]. However, in a trial that allowed flexible dosing of budesonide or prednisone over two years to maintain clinical quiescence and examined bone mineral density (BMD) in relation to efficacy and side effects in CD patients, only 37% of budesonide-treated patients withdrew from the study because of failure to improve or worsening disease. However, the average dose of budesonide required to maintain remissions was higher than (6.8 mg/d) doses used in the placebo-controlled trials. Nevertheless, among patients who were steroid-naïve prior to entering the study, smaller reductions in BMD were seen in the budesonide group compared to the prednisolone group (mean, -1.04% *vs* -3.84%; $P = 0.0084$)[61].

Budesonide at doses below 6 mg/d has been demonstrated to be safe for long-term (one year) use. Results from a pooled analysis of five one-year controlled trials using budesonide 6 mg/d showed that while the overall number of adverse events were not different between the budesonide and placebo groups, patients treated with budesonide had more endocrine and "resistance mechanism" disorders (infection) ($P = 0.0042$ and $P = 0.042$, respectively). The higher incidence of endocrine problems was primarily driven by acne and moon facies, while viral infections accounted for the difference in infection rate. Serious adverse events were reported as rare[62].

In summary, while budesonide is an effective and safe medication for the induction of remission in patients with mild-moderate ileal and proximal colonic disease, optimal dosing schedules to maintain remissions have yet to be established. While budesonide > 6 mg/d or an adjustable dose may maintain remission, a randomized controlled trial is needed to confirm the results of the open-label studies.

IMMUNOMODULATORS

Azathioprine (AZA)/6-Mercaptopurine (6-MP)

6-MP and its prodrug AZA are purine analogs that are converted into 6-thioguanine nucleotides (6-TG); the therapeutically active metabolites interfere with nucleic acid synthesis, exhibit anti-proliferative effects on activated lymphocytes and, most recently, have been shown to induce apoptosis[63,64]. These agents have been studied for the treatment of CD since the late 1960s, with multiple uncontrolled trials showing favorable results. A meta-analysis of AZA and 6-MP for the induction of remission included eight randomized placebo controlled trials ($n = 425$) while another for maintenance of remission included five trials ($n = 319$); three trials with induction and maintenance arms were included in both analyses[65,66]. For active disease, the overall response rate was 54% for patients receiving treatment compared to 33% for those on placebo, yielding a pooled odds ratio (OR) of 2.36 and the number needed to treat (NNT) for one patient to respond was 5; for quiescent disease, overall remission was seen in 67% of patients on treatment compared to 52% of those on placebo, for an OR of 2.16 and NNT of 7. In active disease, those receiving AZA or 6-MP for $\geqslant$ 17 wk resulted in an increased pooled OR of 2.51 and decreased NNT to 4. No dose effect was seen for active disease, but in the maintenance analysis, the OR increased from 1.2 for those taking 1 mg/kg per day to 4.13 at 2.5 mg/kg per day. Fistula healing in the induction studies (defined as complete closure or decreased drainage) was not reported consistently and numbers were small, but a response rate of 55% for treatment compared to 29% for placebo was seen, with an OR of 4.58. One study that was not included because number of fistulae rather than number of patients with fistulae were reported also showed favorable results: 9/29 fistulae (31%) in patients treated with 6-MP compared to 1/17 (6%) in patients taking placebo closed completely[67]. Steroid sparing effects were seen in both the induction and maintenance meta-analyses, with an OR of 3.86 and 5.22 respectively. Patients under treatment for both active and quiescent disease were also more likely to suffer an adverse event leading to withdrawal from studies, with an OR of 3.01 and 4.36 respectively; these events were typically nausea, allergic reactions including fever and rash, pancreatitis and leukopenia. From these studies, it can be concluded that AZA and 6-MP are effective in both the induction and maintenance of remission for CD, although given that maximal clinical benefit may not be evident for three to four months, use of this medication in active disease is best initially coupled with another induction regimen such as steroids, and further, dosing should be optimized for long-term care.

Candy & Wright conducted what is probably the most cited study included in these meta-analyses and elucidates both of these points. Sixty three patients with

active CD were administered a three month taper of prednisolone while randomized to receive either AZA (2.5 mg/kg) or placebo. Although there was no difference in the number of patients achieving remission at wk 12, 42% of the AZA group compared to 7% of the placebo group were in remission at 15 mo ($P = 0.001$)[68]. Further, several studies have evaluated the maintenance benefits of AZA in "withdrawal" trials. In a 12 mo open trial in which 29 patients in remission on AZA for more than two years (median 37 mo) were randomized to continued AZA or withdrawal of AZA, 11/13 (85%) of patients who continued treatment remained in clinical remission compared to 7/15 (47%) of patients who had not continued AZA ($P = 0.043$). This difference was amplified when a subgroup analysis of patients treated with AZA > 1.6 mg/kg per day was performed: 89% of those continued on AZA remained in remission compared to 33% of those withdrawn from AZA ($P = 0.017$)[69]. A larger, longer randomized, controlled trial enrolled patients who had been maintained in remission on AZA for ⩾ 42 mo. Forty patients were randomized to continue the same dose of AZA and 43 to receive placebo for 18 mo. At the end of the study, three patients in the AZA group compared to nine in the placebo group had relapsed: the hypothesis that placebo was inferior to AZA was not rejected ($P = 0.195$). The authors concluded that for patients maintained in remission on AZA, medication should be continued beyond 3.5 years[70].

There is also expanding evidence that AZA is effective as a post-operative maintenance therapy. In an open-label study, 142 patients who had undergone limited bowel resection and/or stricturoplasty were randomized to receive either mesalamine 3 g/d or AZA 2 mg/kg per day within 2 wk of surgery for 24 mo. While risk of clinical (28% *vs* 17% respectively, $P = 0.2$) or surgical (10% *vs* 6% respectively, $P = 0.5$) relapse was equivalent between the two groups, AZA was more effective in preventing clinical relapse among those patients who had undergone more than one surgery for CD (36% *vs* 13%, $P = 0.03$). In this study, adverse events occurred more frequently in AZA-treated patients and caused more frequent study withdrawal (22% *vs* 8%, $P = 0.04$)[71]. In a double-blind, double-dummy multi-center trial, 131 patients who had undergone ileocolonic resection were randomized to daily 6-MP 50 mg, mesalamine 3 g or placebo and were assessed clinically, endoscopically and radiologically at regular intervals over 24 mo. 6-MP was superior at preventing clinical relapse (77%) *vs* mesalamine (58%) or placebo (50%) ($P = 0.045$ for 6-MP *vs* placebo) and endoscopic recurrence (63%, 63%, 43% respectively, $P = 0.03$ 6-MP *vs* placebo) over two years[22].

Thus, treatment with AZA or 6-MP is usually of an "indefinite" duration for patients who have responded. A recent, large European retrospective review of patients treated long-term with AZA demonstrated that in patients with CD, risk of relapse was not greater in patients who discontinued therapy after three to four years, although treatment beyond this time frame improved clinical activity and decreased steroid requirements. The authors conclude that for asymptomatic, steroid-free patients, it may be reasonable to consider discontinuing medication after three to four years of treatment[72].

While thus far treatment with 6-MP or AZA has often been reserved for patients who have required steroids on more than one occasion, there may be benefit to starting these medications earlier in the disease course. A pediatric study randomized children with CD diagnosed within the previous 8 wk to receive 6-MP or placebo for 18 mo, each given with concomitant prednisone. Similar to the Candy study the short-term remission rates were not different between the groups, although patients in the placebo group relapsed significantly more than the 6-MP group (47% *vs* 9%, $P = 0.007$) and required more steroids and for a longer duration[73].

Increased risk of lymphoma with 6-MP and AZA has been debated, with discrepant findings among large series. A recent meta-analysis of six studies ($n = 3891$) showed a four-fold increased risk of lymphoma in IBD patients treated with 6-MP or AZA as compared to the general population: this translated to needing to treat over 4300 patients aged 20-29 and 355 patients aged 70-79 to cause one additional case of lymphoma per year. It is unknown whether this risk relates directly to the medication or to the severity of the disease[74]. Increased risk of hematologic malignancies has also been associated with prolonged leucopenia in IBD patients on 6-MP[75], and EBV-positive lymphomas have also been found more frequently in patients exposed to 6-MP or AZA[76]. The risk of infection with these medication ranges between 0.3%-7.4%[77] and include herpes viruses, human papilloma virus and upper respiratory infections. Physicians prescribing 6-MP and AZA should understand how thiopurine methyltransferase (TPMT) activity affects metabolism of these drugs and should monitor for potential leukopenia and/or hepatotoxicity on a quarterly basis. Measurement of the active metabolite 6-TG may be useful in guiding dosage of these medications.

Methotrexate (MTX)

Methotrexate is a folate analog and reversible competitive inhibitor of dihydrofolate reductase (DHFR). Methotrexate interferes with DNA synthesis and also has multiple anti-inflammatory effects including decreased pro-inflammatory cytokine production and lymphocyte apoptosis[78]. Two exploratory, open-label trials in medically-refractory CD patients with oral[79] or intramuscular (IM)[80] MTX led to the large, multicenter study by Feagan *et al*, in which 141 steroid-refractory patients with active CD were randomized to MTX 25 mg or placebo, intramuscularly over 16 wk. Prednisone was stabilized at 20 mg/d and subsequently tapered over 10 wk. After four months, 39.4% in the MTX group compared to 19.1% in the placebo group had achieved remission (CDAI ⩽ 150 and discontinuation of steroids)[81]. Patients taking MTX suffered significantly more adverse events than the placebo group (16/94) leading to study withdrawal in 17% compared to 2%, although the majority of these side effects were either nausea or asymptomatic liver test abnormalities[81]. Two smaller randomized controlled trials in patients with chronic active disease that compared oral

MTX (12.5 and 15-22.5 mg/wk) did not demonstrate differences in remission rates[82] or flares[83]. More than likely, these unfavorable results are attributable to low, oral dosing with smaller sample sizes as compared to the larger trial. Indeed the bioavailability of oral MTX has been shown to have great variability, averaging 73% that of subcutaneously administered medication[84]. Retrospective data have also reported comparable remission rates to those of Feagan[85-87]. When compared to AZA (2 mg/kg per day) or 6-MP (1.5 mg/kg per day), MTX (25 mg IM changed to po after 3 mo or 15 po/wk) yielded equal rates of remission[88,89], and oral MTX (15 mg/wk) resulted in higher remission rates than 5-ASA 3 g/d (80% *vs* 14%, $P < 0.01$)[89].

MTX also maintains remission in CD. Seventy-six patients who achieved remission with MTX 25 mg IM were randomized to MTX 15 mg IM/wk or placebo. At wk 40, 65% of the MTX group were still in remission as compared to 39% of those in the placebo group and fewer patients required prednisone (28% *vs* 58%, $P = 0.01$). There were no serious adverse events and only one withdrawal from the study secondary to nausea[90]. Several retrospective studies have shown comparable rates patients maintained in remission with MTX[85-87,91].

Mycophenolate mofetil

Mycophenolate mofetil is an ester prodrug of mycophenolic acid which not only inhibits synthesis of guanosine nucleotides and thereby indirectly interferes with T- and B-cell activity, but also inhibits growth of intestinal smooth muscle and synthesis of fibronectin and thus, theoretically could decrease stricture formation. A randomized controlled trial comparing mycophenolate mofetil to AZA in 70 steroid-dependent CD patients with moderately active disease showed equivalent response rates but those with highly active disease seemed to benefit more from mycophenolate mofetil than AZA[92]. Smaller non-randomized studies or series have yielded a combined response rate of 52% overall and 69% in patients with perianal disease[93].

Tacrolimus

Tacrolimus is a macrolide antibiotic used primarily to prevent allograft rejection in the transplant setting. Similar to cyclosporine, it binds to calcineurin and suppresses transcription of activated T-cells leading to decreased pro-inflammatory cytokines such as IL-2, TNFα and INFγ as well as inducing T-cell apoptosis, modifying expression of IL-10 and TGFβ, and may have local effects on the intestine. In a recent review that pooled data from 22 studies with a combined total of 286 patients who had been treated with tacrolimus, promising results in fistulizing disease, unresponsive CD and UC as well as extra-intestinal manifestations were reported[94].

BIOLOGIC AGENTS

Infliximab

Infliximab (Remicade® Centocor, Malvern PA) is a chimeric (75% mouse/25% human) anti-TNFα monoclonal antibody; TNFα mediates multiple pro-inflammatory processes central to the pathogenesis of IBD. The first study that defined efficacy of infliximab in the treatment of active CD randomized patients with moderate-severe, medically-refractory, disease to receive a single infusion of placebo or 5, 10 or 20 mg/kg of infliximab. Seventeen percent, 81%, 50% and 64% of patients respectively had a response (CDAI decrease ⩾ 70 points) at wk 4 ($P < 0.001$ for all infliximab patients *vs* placebo). Overall, 33% of all infliximab patients compared to 4% of placebo achieved remission at wk 4 ($P = 0.005$). While significantly more infliximab patients maintained a response at 12 wk, 37% had relapsed, suggesting that a single dose was insufficient[95]. Those patients who had an initial response to the single infusion were subsequently randomized to receive continued dosing with 10 mg/kg every 8 wk or placebo. After 44 wk, 53% of the infliximab group were in remission compared to 20% of the placebo group ($P = 0.013$)[96].

The ACCENT I study expanded on the potential maintenance benefits of infliximab after an initial response. In the trial, 573 patients received a 5 mg/kg intravenous (Ⅳ) infusion of infliximab at wk 0, after which they were assessed for clinical response by CDAI (decrease in score ⩾ 70 and a 25% reduction in total score). Three hundred and thirty five patients (58%) met this criterion and were randomized to one of three treatment groups: placebo at wk 2 and 6 and then every 8 wk (group Ⅰ), infliximab 5 mg/kg on the same schedule (group Ⅱ) or 5 mg/kg at wk 2 and 6 followed by 10 mg/kg every 8 wk (group Ⅲ). Treatment was continued for 46 wk. At wk 14 or later, patients in all groups who initially had response and then worsened were allowed to cross over to active episodic retreatment (infliximab 5, 10 or 15 mg respectively for groups Ⅰ, Ⅱ, and Ⅲ given on an "as needed" basis). At wk 30, 21% of patients in group Ⅰ, 39% in group Ⅱ ($P = 0.003$) and 45% in group Ⅲ ($P = 0.0002$) respectively were in remission, while median time to loss of response was reported as 19, 38 ($P = 0.002$) and more than 54 wk ($P = 0.0002$) respectively. Significantly more patients in groups Ⅱ and Ⅲ combined (29%) compared with group Ⅰ (9%) had discontinued steroids at wk 54, and fewer hospitalizations and surgeries related to CD occurred in the maintenance therapy groups. There were no differences in serious adverse events between the three groups[97]. A recently published endoscopic sub-analysis of the ACCENT Ⅰ trial showed that scheduled maintenance therapy compared to episodic treatment resulted in greater improvement in mucosal ulceration and higher rates of mucosal healing although the correlation between clinical and endoscopic responses was weak[98].

Infliximab is also effective in the treatment of fistulizing CD. In an initial induction trial, 94 patients with actively draining perianal or abdominal fistulas were randomized to receive three infusions at 0, 2, and 6 wk of placebo, 5 or 10 mg/kg infliximab. Twenty six percent, 68% and 56% of patients respectively achieved reduction in drainage from greater than 50% of fistulas ($P = 0.002$ and $P = 0.02$). Only 13% on placebo compared to 55% and 38% of patients on infliximab had closure of all fistulas ($P = 0.001$ and $P = 0.04$)[99]. In the ACCENT Ⅱ study, 306 patients with one or more draining abdominal or perianal fistulas (⩾ three months duration) received an induction regimen of three infliximab infusions (5 mg/kg).

One hundred ninety-five patients with a response at wk 10 and 14 as well as 87 with no response were randomized to placebo or infliximab (5 mg/kg) every 8 wk to wk 54. Time to loss of response was significantly longer for patients in the infliximab group than placebo (> 40 *vs* 14 wk, $P < 0.001$). Furthermore, at wk 54, 36% in the infliximab group compared to 19% in the placebo group had no draining fistulas ($P = 0.009$)[100]. Relapse of perianal disease after cessation of infliximab may occur earlier than in patients with luminal disease[101].

Antibodies to infliximab are known as both ATIs or HACAs (human anti-chimeric antibodies), and have been associated with lower serum drug concentration levels[97,102] and in turn, with decreased efficacy with episodic treatment[102,103]. In the ACCENT I population, however, equal numbers of antibody-positive and negative patients maintained clinical responses[97]. Additionally, although ATIs are also associated with an increased risk of transfusion reactions[102,103], most ATI positive patients will not have a reaction after re-treatment with infliximab and therefore ATI should not be routinely tested in the absence of loss of response or an infusion reaction[104]. Risk of antibody formation may be decreased by the three-dose induction followed by maintenance therapy[97,103], concomitant use of steroids and/or immunomodulators[97,99,102,103], and pretreatment with hydrocortisone[103]. Sex, location of disease, and smoking status does not appear to correlate with development of ATI[102].

Approximately 30% of patients have no response to infliximab and not all responders have a complete response. As reviewed by Rutgeerts and colleagues, positive predictors of response include elevated CRP, non-stricturing and pure colonic disease subtypes, and concomitant use of immunomodulators[105]. AZA or 6-MP are the immunomodulators most commonly paired with infliximab for CD and it is not clear if the higher response rates seen in combination therapy compared to infliximab alone represents an effect of decreased antibody formation alone or combined efficacy *via* other mechanisms. In contrast to IBD, infliximab has been used concomitantly with MTX in rheumatoid arthritis and a small pilot CD study showed that MTX dosed concomitantly with infliximab may increase remission rates, speed time to remission and decrease steroid use as compared to infliximab monotherapy[106]. Smoking has been found to be a negative predictor of response in two studies[107,108], but surprisingly not in one of the larger studies to examine factors influencing response to infliximab[109].

There has been considerable debate as to whether duration of infliximab treatment must necessarily be life-long or "indefinite," or whether episodic treatment may be a viable alternative. While the clinical and endoscopic benefits of maintenance therapy are demonstrated by ACCENT I and Ⅱ, it has been proposed that the traditional three-dose infliximab induction regimen 0, 2, and 6 wk could serve as a bridge to AZA, but this strategy appeared effective for only six to twelve months[110]. Thus, currently infliximab continues to be recommended for an indefinite period. Another emerging debate is how to position infliximab in the Crohn's treatment algorithm since current regulatory approvals have reserved indications for infliximab for patients who are steroid-refractory or dependent despite immunomodulator therapy. Some argue that this pyramid should be turned upside-down to position infliximab closer to the top, as it has been demonstrated that in steroid-free patients, initial treatment with infliximab and AZA compared with steroids and later addition of AZA leads to significantly more patients in remission and off steroids at 26 wk (60% *vs* 41%, $P = 0.03$) and mucosal healing[111]. If treating early in the disease course with infliximab proved to be disease-modulating, then the "top-down" approach could prove to be the better option to treat those patients on the brink of needing steroids. Arguments against this strategy include the economic costs and the possible safety risks[112].

The safety of infliximab also remains a significant concern with potential serious adverse events including infusion reactions, opportunistic infections including tuberculosis, non-Hodgkin's lymphoma (NHL) and other malignancies, as well as death. A true risk has been difficult to calculate, since most clinical trials did not have continuous placebo arms but instead, were cross-over designs or employed episodic treatment regimens such that most patients were exposed to infliximab at some point[113]. Serious infections were reported in 4% of patients overall in ACCENT I [97] and infliximab-related infections were seen in 8% of a large Mayo ($n = 500$) cohort study of infliximab-treated patients, half of which were serious[114]. As of February 2005, 709 cases of reactivated TB had been reported with infliximab, including 62 deaths[105]. The risk of lymphoma and other malignancies has been difficult to elucidate. The ACCENT I and the Mayo cohort study reported extra-colonic malignancy rates of 1% and 1.5% respectively, but a causal link to infliximab is unclear. CD patients overall likely have a slightly higher risk of NHL[115,116] and squamous cell cancer[116,117].

The TREAT registry has enrolled over 6000 patients from community and academic practices who have been classified in two groups: those who had received infliximab and those who had been treated only with other therapies. The infliximab and non-infliximab patients had similar risks of death, lymphoma and other malignancies; risk of serious infection was slightly higher in the infliximab-treated patients but Cox proportional hazard analysis later found that this risk was independently associated with steroid and narcotic use[118]. In contrast, a recently published decision analytic model projected a slightly increased rate of lymphoma and death in those treated with infliximab compared to those treated with standard therapy, although more quality-adjusted life years were demonstrated in the infliximab group[119]. Twelve cases of hepatosplenic T-cell lymphoma, a rare and incurable type of lymphoma, have been reported in a largely pediatric population (ages 12-31) on combination infliximab and 6-MP or AZA therapy; this association has led to a heightened concern for using these medications concomitantly especially in children, and studies are ongoing to better understand efficacy and safety issues with regard to combination *vs* single agent therapy.

Adalimumab

Adalimumab (D2E7, Humira®; Abbott Laboratories,

Chicago, IL) is a subcutaneously administered recombinant human IgG_1 monoclonal antibody that binds with high specificity and affinity to human TNFα and consists of human-derived heavy and light chain variable regions and human IgG_1 constant region. Adalimumab is now approved in the US and Europe for the treatment of CD. Two open-label trials treated patients with adalimumab who had previous exposure to infliximab. In the first, 24 patients who had lost responsiveness or developed intolerance to infliximab were treated with an initial dose of adalimumab 80 mg and then 40 mg every other week for 12 wk. Although 79% required dose escalation to 40 mg weekly, clinical remission and response at wk 12 was seen in 29% and 59% respectively[120]. In the second trial, 15 patients with attenuated response to infliximab were treated for six months with the same schedule of adalimumab as in the first study. Of the 13 patients who completed the trial, 54% had a complete response, 31% had a partial response, and 73% were able to discontinue steroids[121]. Most recently, the CLASSIC- I trial randomized 299 moderate to severe CD patients naïve to anti-TNF therapy to one of three dose combinations administered at wk 0 and 2 (160/80 mg, 80/40 mg, or 40/20 mg) or placebo. At wk 4, 36% (P = 0.001), 24% (P = 0.06), and 18% (P = 0.36) in the adalimumab groups, respectively, were in clinical remission compared to 12% in the placebo group[122]. Fifty-five patients who were in remission at wk 4 of CLASSIC I were randomized to receive continued adalimumab 40 mg every other week, weekly or placebo for up to one year as part of the CLASSIC Ⅱ trial in which 74%, 83% and 44% of patients, respectively, maintained remission at wk 56[123]. Similar to the ACCENT I study with infliximab, immunomodulator therapy again did not alter these results[124]. Finally, the CHARM trial (n = 854) examined adalimumab induction and maintenance efficacy in patients with moderately to severely active CD. An 80 mg dose at week zero and 40 mg dose at wk 2 were administered to all patients, with 499 (58%) achieving clinical response and then randomized to placebo, adalimumab 40 mg every other week, or 40 mg weekly through wk 56. Significantly higher rates of remission were seen in the adalimumab groups compared to placebo at both wk 26 (40% and 47% *vs* 17%, $P < 0.001$) and wk 56 (36% and 41% *vs* 12%, $P < 0.001$). The adalimumab groups also had significantly more steroid discontinuation and complete fistula closure. Safety data was comparable to other TNF therapy[125].

Certolizumab

Certolizumab pegol or CDP870 (UCB; Smyrna, GA) is a monoclonal humanized anti-TNFα antibody Fab' fragment linked chemically to polyethylene glycol (PEG). In contrast to infliximab and adalimumab the antibody fragment does not induce apoptosis[126]. Certolizumab has been evaluated in both induction and maintenance trials for CD[126,127]. In 92 patients with moderate to severe CD randomized to a single intravenous dose of 1.25, 5, 10 or 20 mg/kg of CDP870 or placebo, the primary endpoints of clinical response or remission after four weeks were not different between treatment groups and placebo, but the remission rate at wk 2 was 47% in the 10 mg/kg group compared to 16% in the placebo group (P = 0.041)[127]. The PRECISE 1 study compared subcutaneous certolizumab (100, 200 or 400 mg) to placebo administered at wk 0, 4, and 8 in 292 patients with moderate-severe CD. While all doses of certolizumab produced significant clinical benefit over placebo at wk 2, 400 mg had the strongest effect at all time points, most markedly at wk 10 (52.8% *vs* 30.1%, P = 0.006); however, no statistical significance in clinical response was seen at wk 12, the primary endpoint. When re-analyzed according to stratification by C-reactive protein level (> 10 mg/L), the 400 mg group had a significantly better response at wk 12 (53.1% *vs* 17.9%, P = 0.005) that was attributed to a lower placebo response rate than those patients with a CRP < 10[126]. In the PRECISE 2 trial, patients who responded to a 400 mg induction dose at wk 0 and 2 (428/668, 64%) were randomized to receive 400 mg certolizumab or placebo every 4 wk for 26 wk. Significantly more patients in the certolizumab arm achieved clinical response (62.8% *vs* 36.2%, $P < 0.001$) and remission (47.9% *vs* 28.6%, $P < 0.001$) at wk 26[128]. Safety and tolerability were similar to other anti-TNF agents, although patients treated with certolizumab had lower rates of autoantibody formation.

Fontalizumab

Interferon γ is cytokine with wide-ranging proinflammatory activity implicated in both animal models of colitis and found to have mucosal elevations in CD. Fontalizumab (Protein Design Labs Inc, Fremont, CA, USA) is a humanized form of mouse antihuman interferon γ antibody recently studied in CD. A controlled trial randomized 133 patients with moderate-severe CD to receive one or two doses of fontalizumab 4 mg/kg, 10 mg/kg, or placebo (28 d apart). Although no differences in response were demonstrated with single dose therapy, in those receiving two doses, response rate at d 56 was found to be 69% and 67% for the fontalizumab groups compared to 32% in the placebo groups (P = 0.02 and 0.03 respectively). This difference was more robust in patients with elevated CRP. Adverse rates were similar across treatment and placebo groups, and all serious adverse events except one were related to CD exacerbations[129].

SELECTIVE ADHESION MOLECULE INHIBITORS

Leukocyte emigration from the vascular space to inflamed tissue is a complicated process involving multiple leukocyte-endothelial interactions including tethering, rolling, firm adhesion, spreading, and migration. Leukocyte adhesion to activated endothelium is mediated primarily by the α_4 and β_2 integrins. The α_4 integrin is expressed on all types of white blood cells and can pair with either the β_1 or β_7 subunit. Endothelial ligands recognized by α_4 integrin include vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MadCAM-1); the former is induced at sites of inflammation, whereas the latter is expressed constitutively on the endothelium within Peyer's patches and other gut-associated lymphoid tissues[130].

Natalizumab

Natalizumab is a recombinant humanized antibody derived from a murine monoclonal antibody (AN100226m) (95% human and 5% mouse-derived) and targets human α_4 integrin. Antibodies to α_4 integrin have shown efficacy in animal models of multiple sclerosis and colitis[131,132]. The preliminary data supporting the use of natalizumab as an induction agent was equivocal as two trials of natalizumab in CD showed a trend toward clinical benefit with either one or two doses of 3 or 6 mg/kg compared to placebo, but primary endpoints did not reach significance[133,134]. Similarly, ENACT-1 did not demonstrate significant differences in clinical response or remission at 10 wk between CD patients (n = 905) treated with an intravenous infusion (300 mg) of natalizumab and placebo at wk 0, 4, and 8[135]. In contrast, the ENCORE study enrolled only patients with an elevated CRP (n = 509) and showed significantly higher rates of clinical response and remission at all time points in those treated with three doses of 300 mg natalizumab (0, 4 and 8 wk) compared to placebo[136].

More consistent outcomes have been shown in maintenance trials for natalizumab. In ENACT-2, initial responders to natalizumab (n = 339) received natalizumab (300 mg) or placebo every 4 wk through wk 56. Significantly more patients in the treatment group compared to placebo had a sustained response (61% *vs* 28%, $P < 0.001$) and remission (44% *vs* 26%, $P = 0.003$) through wk 36[135] and concomitant use of immunomodulators did not affect efficacy[137]. An open-label extension study of ENCORE showed that 84% of patients who were in remission after one year remained in remission for two years after continued monthly treatment with natalizumab[138].

In early 2005, three cases of progressive multifocal leukoencephalopathy (PML) were reported in patients treated with natalizumab, two of them fatal. Two patients had multiple sclerosis and one had CD. Natalizumab trials were subsequently suspended by the U.S. Food and Drug Administration (FDA) and the drug was removed from the market. A safety trial that included 90% of all CD, multiple sclerosis and rheumatoid arthritis participants from all previous natalizumab clinical trials failed to find any additional cases of PML, and the overall risk of PML was estimated at 0.1%[139]. In June 2006, the FDA approved resumption of natalizumab marketing targeting a restricted distribution program for selected MS patients.

MLN02

MLN02 is a humanized monoclonal antibody which specifically recognizes the α_4B_7 heterodimer but does not cross-react with the individual component monomers[140]. The major ligand for α_4B_7 is MadCAM1, and therefore this antibody should be gut-specific in theory. Although clinical and endoscopic efficacy has been demonstrated in patients with UC, patients with mild to moderate CD (n = 185) who received two doses of either 0.5 or 2.0 mg/kg at 0 and 29 d did not achieve the primary endpoint of clinical response at two months. On the other hand, clinical remission was seen in 36.9% of the 2.0 mg/kg group compared with 20.7% of the placebo group ($P < 0.05$)[141].

OTHER BIOLOGIC AGENTS

Visilizumab

Visilizumab (Nuvion™, Protein Design Labs) is a humanized IgG_2 monoclonal antibody (HuM291) to the CD3ε chain of the T-cell receptor expressed on activated T-cells. Designed to capitalize on the potent immunosuppressive effect of OKT3 (a mouse monoclonal antibody used primarily in the transplant setting), it minimizes the anti-mouse antibody response and also the adverse effects of the cytokine release syndrome. While trials in UC have demonstrated safety and efficacy, only one small open-label trial in medically-refractory (including infliximab) CD patients (n = 14) given two doses of visilizumab10 μg/kg on d 0 and 1, 58% and 33% experienced clinical response and remission respectively on d 89, with the mean prednisone dose dropping from 19 mg/d at baseline to 4 mg/d[142].

Anti IL-6 receptor antibody

IL-6 is another cytokine that plays a central role in the inflammatory process of CD. A monoclonal antibody to IL-6 receptor (IL-6R) has been shown to decrease expression of adhesion molecules and multiple pro-inflammatory cytokines in animal models of colitis. A randomized pilot study of humanized anti IL-6R (MRA) in 36 patients with active CD found that those given a biweekly infusion of MRA had an 80% clinical response rate compared to 31% of placebo patients ($P = 0.019$), although endoscopic and histologic examination showed no differences[143].

Anti IL-12 antibody

Interleukin-12 is an important cytokine in the Th1-mediated inflammatory response. A monoclonal antibody targeting IL-12 has been evaluated in a randomized trial in which uninterrupted weekly dosing at 3 mg/kg for seven weeks yielded higher response rates than placebo (75% *vs* 25%, $P = 0.03$), but a statistically significant difference was lost at 18 wk (69% *vs* 25%, $P = 0.08$). The more robust clinical response in the anti-IL-12 group was paralleled by decreases in colonic mononuclear cell secretion of IL-12, INF-γ, and TNFα[144].

Thalidomide

Because of its anti-TNFα and anti-IL-12 properties, thalidomide has been studied in two small open-label trials in mixed IBD populations, with the majority of patients in each achieving either clinical response or remission[145,146]; use of this medication is severely restricted because of its well-known teratogenicity and it is further limited by side effects of sedation and mood disturbances.

IMMUNE STIMULATION

Although immune dysregulation is believed to be a part of the pathogenesis of IBD, an alternative hypothesis proposes that an altered innate immune response is inherent to the etiology of CD. Based upon positive results in other disorders of neutrophil function, granulocyte-

macrophage colony-stimulating factor (GM-CSF), a myeloid growth factor that stimulates the growth and function of phagocytic cells, has been studied in CD. One hundred and twenty-four patients were randomized to receive sargramostim (GM-CSF) 6 mcg/kg per day subcutaneously or placebo for 8 wk: while the primary endpoint of a clinical response (defined by a decrease in CDAI of $\geqslant$ 70 points) was not met, significantly more patients in the sargramostim group reached the secondary endpoints of a decrease in CDAI of $\geqslant$ 100 points (48% *vs* 26%, $P = 0.01$) and remission at d 57 (40% *vs* 19%, $P = 0.01$). The sargramostim group suffered from significantly more injection site reactions and experienced more bone pain[147].

PROBIOTICS AND HELMINTHS

The theory of dysbiosis maintains that a decrease in protective or "good" bacteria and a concomitant increase in harmful or "bad" bacteria contribute to the pathogenesis of IBD. As a result, probiotics have been studied as both induction and maintenance treatment in CD. Open label and small randomized controlled trials using various preparations of probiotics have shown inconsistent results as summarized by Rioux and Fedorak[148]. Large randomized placebo controlled trials are needed in order to determine true efficacy. Similarly, observations that IBD is uncommon in developing countries where helminthic colonization is prevalent and that helminths downregulate Th1 immune responsiveness have led to trials utilizing non-pathogenic helminthes in an attempt to treat UC and CD. An open-label trial of *Trichuris suis* (porcine whipworm) has been studied as a therapy for CD in which 29 patients with active CD ingested 2500 live *Trichuris suis* ova every 3 wk for 24 wk: as defined by CDAI scores, 79% responded and 72% remitted[149]. While results from this study are intriguing, a controlled trial again is essential before deeming worm therapy beneficial.

ULCERATIVE COLITIS

Aminosalicylates

Aminosalicylates (5-ASA) remain the first-line therapy for both induction and maintenance of mild-moderate UC. The efficacy of sulfasalazine specifically has been well-established, but dose-dependent intolerance to the sulfa moiety limits its use in up to one-third of patients[150]. Therefore, non-sulfa-containing 5-ASA agents have been studied as well. Recent Cochrane reviews analyzed the effectiveness of these newer 5-ASA medications both in comparison to placebo and sulfasalazine for the induction and maintenance of remission in UC. Twenty one randomized controlled trials ($n = 2124$) of 5-ASA were included in the induction meta-analysis, nine comparing 5-ASA to placebo and 12 to sulfasalazine[151]; results were reported in terms of failure rates. 5-ASA provided benefit over placebo in the induction of remission, with a pooled OR of 0.53 overall and 0.36 when only the Asacol trials were included. While 5-ASA was better than placebo across all dosage ranges, there was a trend toward a dose-effect. 5-ASA was also more likely to elicit a global or clinical response than placebo, with a pooled OR of 0.40 and higher doses yielding better results, $P = 0.002$. 5-ASA was also superior to placebo at inducing endoscopic remission, but only at doses $\geqslant$ 3 g/d. No significant differences were found between 5-ASA and sulfasalazine in induction of remission or response, although a trend towards 5-ASA superiority was observed. Significantly more patients taking sulfasalazine withdrew from studies secondary to adverse events, with an OR of 0.34; it should be noted that tolerance to sulfasalazine was an inclusion criteria for most of the studies, which may have made this effect less robust.

Sixteen trials ($n = 2479$) were included in the 5-ASA maintenance of remission meta-analysis, five comparing 5-ASA to placebo and 11 to sulfasalazine[152]. 5-ASA was more effective than placebo in maintaining endoscopic or clinical remission, with an OR of 0.47; a dose effect was not observed. Sulfasalazine was superior to 5-ASA in the maintenance of remission in trials of six month duration, with an OR of 1.29, but the statistical significance was lost when only studies with endpoints at 12 mo were included. In subgroup analyses by specific 5-ASA preparation, only olsalazine was found inferior to sulfasalazine, likely secondary to the greater number of adverse events (the most common being diarrhea) and subsequent withdrawals in patients receiving this medication. The authors stated that conclusions could not be reached with regard to other 5-ASA preparations. Save for olsalazine, there were no differences in adverse events between 5-ASA when compared to placebo or sulfasalazine. If sulfasalazine truly has superiority over 5-ASA in maintenance of remission (beyond olsalazine), the authors conjecture that unknown pharmacologic effects of the sulfapyridine moiety previously thought to function only as a carrier of 5-ASA to the colon, could contribute to this finding.

5-ASA formulations

There is no definitive data to suggest that one 5-ASA preparation is superior to another. In one study, balsalazide 6.75 g/d (Colazal®, Salix Pharmaceuticals, Morrisville, NC, USA) was found to induce remission in a greater number of patients with active moderate-severe UC than equivalent doses of Asacol 2.4 g/d (62% *vs* 37% at 12 wk, $P = 0.02$). Further, the median time to complete symptom relief was significantly shorter in the balsalazide than mesalamine group (10 *vs* 25 d, $P = 0.004$)[153]. Two subsequent studies comparing these same medications at the same doses did not demonstrate differences in primary endpoints of rectal bleeding and at least one other sign or symptom at wk 8[154] or symptomatic remission at wk 8[155]. Secondary endpoints showing balsalazide to have a faster time to onset[154,155] or better effect in new onset left-sided disease[155] cannot be considered more than preliminary given that primary endpoints were not met[156]. Further, no differences between balsalazide and sulfasalazine or Salofalk® (a delayed-release pH dependent mesalamine formulation) have been found[156]. Additionally, as will become evident below, a suboptimal dose of Asacol® was used in these studies[157]. As well, non-traditional clinical assessments were employed[158], and the Asacol® was not equivalent to that used in the US and in pivotal trials, as demonstrated by *in vitro* dissolution experiments[159].

Pharmacokinetic data in healthy patients demonstrates no differences in systemic absorption of 5-ASA between Asacol and balsalazide at equimolar doses[160]. Therefore, choice of 5-ASA agent should be based upon tolerability, ability to titrate dose to effect and cost.

Dose-effect

The recent ASCEND trial showed a dose-effect based on severity of disease. An overall response rate of 72% at wk 6 was found in patients with moderate activity (n = 268) treated with mesalamine 4.8 g/d (investigational 800 mg tablet, Procter and Gamble Pharmaceuticals, Mason, OH) compared to 59% in those receiving Asacol® 2.4 g/d (P = 0.036). No difference in response rate in patients with mild disease was demonstrated with the two different doses[161]. Patient compliance in taking 5-ASA may be enhanced by a higher dose tablet, SPD476 (1.2 g/tablet) which uses both a gastro-resistant polymer film to delay release of active drug until it reaches the terminal ileum and Multi Matrix System (MMX) which helps deliver 5-ASA evenly throughout the colon. MMX 2.4 g/d and 4.8 g/d are superior to placebo in the induction of remission in mild-moderate UC[162].

Topical mesalamine

Rectal mesalamine induces remission more effectively than placebo or topical steroids in distal UC[163,164], although both medications taken concomitantly are superior to mesalamine alone[164]. Topical mesalamine is superior to placebo and at least as effective as oral mesalamine in the maintenance of remission for distal UC[163,164].

STEROIDS

For patients without sufficient response to 5-ASA agents or those with moderate-severe disease, glucocorticosteroids have remained the foundation for inducing remission in UC since the early 1950s when Truelove and Witts reported significant benefit for cortisone over placebo[165]. For mild to moderate UC, a dose effect for prednisone 20-60 mg/d has been reported, but doses greater than 60 mg/d confer no additional benefit[166]; further, there does not appear to be a difference between once daily and divided dosing[167]. For those with severe colitis or not responding to oral regimens, parenteral steroids are administered. While mineralocorticoid and anti-inflammatory potencies vary, no data suggests one preparation is superior to another; methylprednisolone 40-60 mg/d or an equivalent dose of hydrocortisone is the most commonly used. Adrenocorticotropic hormone (ACTH) promotes endogenous corticosteroid production and may have benefit in steroid-naïve patients, but is no longer commonly utilized due to the potential for adrenal hemorrhage[35]. Pulse-dose steroids in the form of dexamethasone 100 mg/d have shown efficacy in a small open-label trial[168], but a controlled trial has not yet been conducted and a recent systematic review suggests the absence of a dose-response above the equivalent of 40 mg of prednisone[169]. Dividing intravenous bolus dosing is equally effective to a continuous infusion[170].

Predictors of decreased response rate to steroids and increased risk for colectomy include greater severity and extent of colitis[171,172], and most recently persistence of stool frequency > 8/d or CRP > 45 mg/L beyond three days of treatment[173]. Higher levels of glucocorticoid receptor beta (GRβ) have also been associated with glucocorticoid resistance in several studies[174-176]. Of those patients who will respond to IV steroids, the majority do so within five days[177], but most practitioners will continue treatment for 7-10 d[35].

NON-SYSTEMIC STEROIDS

With the multitude of adverse effects of systemic steroids, non-systemic steroids have generated great interest in UC given their high-first pass metabolism and minimal toxicity. Because most of budesonide is released in the distal ileum and proximal colon, making it an effective medication for the treatment of CD in this location, its role in UC is likely very limited, although one study showed equal efficacy to prednisolone in those with left-sided or extensive colitis[178]. An oral formulation of beclomethasone dipropionate (BDP) coated with Eudragit L preventing gastric dissolution and releasing at pH 6.0 for delivery in the terminal ileum and throughout the colon was evaluated in a single-blind randomized trial enrolling 177 patients with mild-moderate UC[179]. Patients who received BDP 5 mg/d for 4 wk had equivalent reductions in mean disease activity index or DAI (assessment of clinical and endoscopic response) and clinical remission rates as those receiving 5-ASA 2.4 g/d, although a significantly greater improvement in DAI was seen in those with extensive disease in the BDP group. BDP was also found to have an additive effect when given in conjunction with 5-ASA[180].

Non-systemic steroid enemas are beneficial in the treatment of active distal UC. Budesonide enemas have significantly higher remission rates than placebo, between 19%-51% with daily 2 mg/100 cc dosing[181,182]; higher doses do not appear to be of greater benefit but may result in more adrenal impairment[182]. While 2 mg twice weekly was not any more effective than placebo in the maintenance of remission[182], it is possible that the optimal dosage for preventing relapses has not been defined. When compared to topical mesalazine (1 g/100 mL per day), budesonide enemas were equally effective in improving histologic and endoscopic scores but clinical remission rates were higher in the mesalazine group[183]. Budesonide enemas are equally or more beneficial than traditional steroid enemas in clinical, endoscopic and histologic measures and induce less adrenal suppression[184-186]. Budesonide foam and enemas resulted in similar clinical remission rates in a large double-blind, double-dummy trial[187]. Similarly, BDP enemas have shown equal efficacy to 5-ASA[188] and prednisolone enemas[189], but are not associated with adrenal axis suppression[189]. The combination of 5-ASA and BDP was superior to either alone[188].

IMMUNOMODULATORS

AZA and 6-MP

The first reported use of AZA in the treatment of UC was in the 1960s, but results from initial controlled trials

in the mid-1970s did not show clinical or endoscopic benefits over placebo[190,191]. However, it became apparent that treatment with AZA consistently permitted significant steroid reduction compared to placebo[191,192]. Patients with UC in remission on AZA ⩾ six months relapsed at a higher rate over one year when withdrawn to placebo (59%) as compared to those who continued AZA (36%), $P = 0.039$; this effect was more pronounced with longer pre-trial remission rates[193]. Later retrospective studies also reported the steroid-sparing effect of AZA and 6-MP[194-197], higher relapse rates with cessation of 6-MP[194,195], and fewer colectomies in those patients maintained on AZA[196,197]. Length of treatment appears to correlate with efficacy: a large retrospective review of both CD ($n = 272$) and UC ($n = 346$) patients treated with AZA found a remission rate of 87% in those patients treated more than six months, compared to 59% overall. Other factors predictive of remission were the diagnosis of UC (*vs* CD), lower white blood cell (WBC) or neutrophil count, a higher mean corpuscular volume and older age. On continued AZA, 95%, 69%, 55% of patients at 1, 3 and 5 years respectively were maintained in remission compared to 63%, 44%, and 35% after discontinuation of AZA; risk of relapse was lower in those with WBC ⩽ 5.0×10^5 ($P = 0.03$)[198].

Compared to mesalazine at a dose of 3.2 g/d, significantly more steroid-dependent patients treated with AZA, 2 mg/kg, achieved both clinical and endoscopic remission as well as steroid discontinuation (53 *vs* 21%, $P = 0.006$)[199]. The addition of 5-ASA to AZA does not confer greater benefit than AZA alone in the maintenance of remission[200,201] or steroid-withdrawal[200].

METHOTREXATE (MTX)

While an initial small open-label study of MTX in IBD held promise for both CD and UC[80], randomized controlled trials have shown benefit in CD only[81,90]. Although several additional open-label or retrospective studies of MTX in UC showed favorable effect[91,202,203], two randomized controlled trials showed no differences in the induction or maintenance[89,204] of remission between patients given oral MTX 12.5[204] or 15 mg[89] per week compared to placebo. It should be noted, however, that in the definitive study that established the efficacy of MTX in the induction of remission in CD, patients were given 25 mg IM; ideally, a second randomized controlled trial in UC utilizing a higher dose of MTX would be conducted.

CYCLOSPORINE

Cyclosporine (CSA) is a lipophilic peptide with multiple anti-inflammatory effects including downregulation of IL-2, thus inhibiting proliferation and activation of T-helper cells[205]. After the first promising open-label trial in 1990 in which 73% of 15 severe, steroid-refractory UC patients treated with Ⅳ CSA (4 mg/kg) improved over an average of 5.8 d and avoided colectomy[206], a double-blind controlled trial that randomized 20 patients with severe steroid-refractory UC to Ⅳ CSA (4 mg/kg) or placebo showed an 82% response rate in the CSA group at a mean of seven days compared to zero in the placebo group ($P < 0.001$). All five placebo group patients given CSA during the open-label phase responded to treatment and over two-thirds of all responders avoided colectomy at six months[207]. In non-randomized controlled trials, long-term remission rates have been less impressive ranging between 14%-40%[208-212]; "bridging" to AZA after induction with Ⅳ CSA and a short course of oral CSA improves maintenance rates, with 40%-90% of patients avoiding colectomy after 16-78 mo[213-215]. Similarly, in a retrospective review at the University of Chicago, 62% of all UC patients ($n = 42$) treated with CSA avoided colectomy over a mean follow-up of 23 mo. This rate improved to 72% among initial CSA responders and to 80% in initial responders who were later transitioned to 6-MP or AZA[216]. Skipping oral CSA and transitioning directly to (6-MP or AZA) after Ⅳ therapy does not seem to alter long-term outcome[217].

CSA is associated with significant morbidity, including opportunistic infections, neurologic and renal toxicity, hypertension, and rarely, death[218]. Several variations in therapy may decrease the risk of these adverse events without compromising efficacy: the use of low-dose CSA (2 mg/kg)[218-220], oral microemulsion CSA (Neoral®) with 60% bioavailability[221-223], and Ⅳ CSA without concomitant steroids[224]. Higher percentages of band forms on differential WBC count[225], tachycardia > 90 bpm, fever > 37.5℃, elevated CRP > 45 mg/L, and greater than one severe endoscopic lesion are negative predictors of response[226]. Ideally, administration of CSA should be limited to physicians trained or experienced in the use of potent immunosuppressants or transplantaion[227].

TACROLIMUS

UC patients with active refractory moderate-severe disease ($n = 63$) were randomized to tacrolimus dosed to maintain either a high (10-15 ng/mL) or low (5-10 ng/mL) trough or placebo for two weeks with an open-label extension segment. Sixty-eight percent of patients in the high-trough group achieved partial response as measured by the UCDAI (number of bowel movements, bleeding and physician's global assessment) compared to 10% in the placebo group ($P < 0.001$). While 38% in the low-trough group achieved a partial response, this did not meet statistical significance; however, significant differences were found on multiple components of the UCDAI between the low-trough group and placebo. When placebo patients crossed-over to the open-label extension, 58% achieved response ($P = 0.012$). While no differences in overall adverse events were found, patients in the high-trough group experienced more medication-related adverse events than the placebo group[228]. A recent review of tacrolimus in IBD patients also found overall favorable results in the treatment of refractory UC[94].

MYCOPHENOLATE MOFETIL

Few trials have examined the efficacy of mycophenolate mofetil (MMF) in UC. In a six month open-label uncontrolled study, 24 steroid-dependent chronic active IBD patients received MMF 2 g/d and were tapered to

5 mg of prednisone per day by the second three months. Among the 13 UC patients, six achieved remission by three months, but all relapsed during the second part of the study[229]. The results were equally disappointing in the CD population, with only one patient maintaining remission by the end of the study. A retrospective study of 39 largely steroid-dependent and AZA-refractory or intolerant IBD patients given a median dose of MMF 1.5 g/d reported more favorable results, with 40% of patients in remission off steroids after a mean duration of 19 mo of treatment[230]. An open-label study randomized 24 patients with active UC to receive either MMF (20 mg/kg) or AZA (2 mg/kg), each given with a tapering dose of prednisolone over one year. While the AZA group experienced significantly greater decreases in the clinical colitis activity index (CAI) than the MMF group at three and six months, these differences were no longer significant at nine and twelve months. Further, although at almost all time points, more AZA-treated patients were in remission and using fewer steroids than the MMF group, none of these differences were statistically significant[231].

BIOLOGIC AGENTS

Infliximab

While several small studies of infliximab collectively showed equivocal efficacy in UC, the ACT (Active Ulcerative Colitis Trials) 1 and 2 provided definitive evidence supporting its efficacy in this population[232]. ACT 1 patients were refractory or intolerant to steroids and/or AZA/6-MP, while ACT 2 also included those refractory or intolerant to 5-ASA agents as well. In each trial, 364 patients received either placebo or infliximab 5 or 10 mg/kg at wk 0, 2, and 6 and then every 8 wk through wk 46 and 22 with follow-up data collected to wk 54 and 30 respectively. In ACT 1 and 2, infliximab at either dose was significantly more beneficial than placebo at all time points in achieving clinical response and remission, mucosal healing, and discontinued use of steroids. Overall, approximately two-thirds in the infliximab group achieved clinical response and one-third achieved long-term remission, while 22% discontinued steroids. Rates of adverse events were similar between groups, although one case each of tuberculosis and histoplasmosis (the latter resulting in death) as well as three neurologic complications occurred in the infliximab group. ATIs were found in 6% and conferred a mildly higher risk of infusion reaction. Concomitant immunomodulator therapy was associated with a lower rate of antibody formation, but no conclusions can be reached given the small numbers with ATI overall. Five mg/kg is the recommended starting dose given that there were no significant differences found between the two doses.

While the ACT studies have established infliximab as effective treatment for UC in the outpatient setting, the role of infliximab in the treatment of hospitalized patients is uncertain. Forty-five moderate-severe or fulminant UC patients refractory to Ⅳ steroids at 5 and 3 d respectively were treated with a single dose of infliximab 5 mg/kg or placebo. Overall, infliximab patients avoided colectomy within the first three months more often than placebo patients (67% *vs* 29%, $P = 0.017$); however, in a subgroup analysis of those with fulminant colitis compared to placebo, this difference was no longer significant (69% *vs* 47%, $P = 0.276$)[233]. In an open-label trial of infliximab in 12 hospitalized steroid-refractory UC patients, nine underwent colectomy within three months[234]. Two recent studies showed favorable response profiles to infliximab in patients with acute severe UC[235,236], and it has been hypothesized that this subset of patients may be different than those with established disease.

SELECTIVE ADHESION MOLECULE INHIBITORS

Natalizumab (Tysabri®, Elan and Biogen Inc, USA)

Only one open-label trial of natalizumab has been conducted in UC, in which 10 patients with active disease were given a single dose of 3 mg/kg: while significant clinical and quality of life improvement were seen at one month, only two patients entered remission and by 8 wk, 80% of patients required rescue medication[237]. Given the association of PML with this medication in CD and MS patients, the status of future trials is unknown.

MLN02

Compared to those who received placebo, mild-moderate UC patients ($n = 181$) who received two doses of MLN02 0.5 or 2.0 mg/kg over one month experienced higher rates of clinical remission (33% and 32% *vs* 14%, $P = 0.03$), clinical response (66% and 53% *vs* 33%, $P = 0.002$), endoscopic remission (28% and 12% *vs* 8%, $P = 0.007$) and endoscopic improvement (48% and 35% *vs*16%, $P = 0.001$)[140]. Antibodies to MLN02 were found in 44% of patients; of those with titers ⩾ 1:125, 24% had loss of saturation to $\alpha_4\beta_7$ binding sites with the clinical remission rate in this group close to that of placebo. There were no differences in adverse events. MLN02 appears promising, but more research will need to assess long-term response and optimal dosing.

Alicaforsen

Alicaforsen (ISIS 2302, Isis Pharmaceuticals, Inc. Carlsbad, CA) is a 20-base phosphorothioate oligodeoxynucleotide antisense molecule that down-regulates messenger RNA for intracellular adhesion molecule I (ICAM-1), a transmembrane glycoprotein that is up-regulated by pro-inflammatory mediators. ICAM-1 is involved in leukocyte activation and migration and elevated levels in serum and mucosa have been found in animal models and patients with IBD[238]. Parenteral alicaforsen was not effective in CD[239], but enema formulations appear beneficial in UC and pouchitis in small studies. In one trial ($n = 40$), mild-moderate active distal UC patients who received daily alicaforsen enemas at 0.1, 0.5, 2, or 4 mg/mL for one month experienced an overall dose-dependent improvement in disease activity index (DAI) ($P = 0.003$). At three months, DAI in the 4 mg/mL group dropped by 72% compared with 11.5% in the placebo group ($P = 0.016$), and no one in the alicaforsen group needed additional therapy at six months compared to 50% of placebo patients[240]. No serious adverse events were reported.

ANTI-INTERLEUKIN-2 (IL-2)

IL-2 is a cytokine produced by activated T-cells that binds to the high affinity receptor IL-2R in the presence of the α-chain CD25 and thereby perpetuates T-cell proliferation and activation[241]. Further, high levels of IL-2 have been associated with steroid-resistance[242]. Two anti-IL2 antibodies have been evaluated in UC.

Daclizumab (Zenapax, Roche, Basel, Switzerland), is a recombinant humanized monoclonal antibody (IgG_1) to IL-2R. Although a small, open-label pilot study initially held promise for this antibody[243], a recently published randomized controlled trial (n = 159) found no difference in response or remission rates between two doses of daclizumab and placebo given every other week for 8 wk[244]. Basiliximab, a chimeric monoclonal antibody to the IL-2R (CD25) α-chain, induced remission in the majority of 10 steroid-resistant UC patients given a single dose in an open-label trial[241]. Additionally, *in vitro* testing performed in healthy volunteers and quiescent UC patients as part of this study showed that basiliximab reverses steroid-resistance, and thus anti-IL-2 treatment might have particular potential in steroid-resistant patients.

VISILIZUMAB (NUVION)

Visilizumab, an anti-CD3 monoclonal antibody is undergoing evaluation in severe UC. In an open-label phase I trial, 79% and 54% of steroid-refractory UC patients treated with 10 mcg/kg per day (n = 24) for two consecutive days experienced response and remission respectively at d 30, and 100% of those treated with 15 mcg/kg per day (n = 8) achieved both response and remission[245]. Sixty-three percent of patients receiving the higher dose remained in remission at one year. Almost two-thirds of patients experienced symptoms of cytokine release syndrome 1-3 h post-infusion, including nausea, chills, fever, headache and arthralgias. Decreased T-cell levels persisted for a mean of three weeks post-infusion. Because elevations in EBV titers were reported in patients with graft versus host disease who received visilizumab[246], this UC study excluded EBV+ patients, but a large open-label trial including EBV+ patients is ongoing.

INTERFERONS

Interferon-alpha (IFNα)

IFNα has a range of anti-viral, anti-tumor and anti-inflammatory activity, including induction of IL-1 receptor antagonist and soluble TNF receptor p55, and downregulation of Th-2 cytokines[247]. With the recognition that IFNα does not induce colitis flares in those being treated for chronic hepatitis who have co-morbid UC[248,249] IFNs have been studied as treatment for UC.

In a small (n = 28) open-label trial of IFNα2a given subcutaneously for 6-12 mo in UC patients, 93% achieved and maintained clinical and endoscopic remission for two years[250], while another small study showed only short-lived benefit of IFNα2a over prednisolone enemas in treating distal UC[251]. In the only randomized placebo controlled trial, UC patients refractory to 5-ASA agents, steroids or AZA (n = 60) received either weekly pegylated INFα2b 0.5 or 1 mg/kg or placebo: no difference in clinical or endoscopic response was demonstrated, and a high attrition rate was seen in all groups, mostly secondary to lack of efficacy[247]. Thus, while INFα does not appear to exacerbate UC when treating chronic hepatitis, it is not effective as a primary treatment for UC.

Interferon B

Interferon β (INF-β) also has anti-inflammatory properties including the upregulation of IL-10 and IL-1 receptor antagonist and downregulation of TNF and IL-2. INF-β has had varied results in the treatment of UC. In an open-label pilot study, 88% of 25 steroid-refractory UC patients treated with either Ⅳ human natural IFN-β or subcutaneous recombinant IFN-β for a mean of 52 wk achieved remission lasting over a year[252]. While one small randomized trial showed endoscopic benefit in patients treated with IFNβ1 compared to placebo[253], a larger controlled trial failed to show any advantage recombinant IFNβ1a over placebo in clinical response or remission, endoscopic index, or steroid reduction[254].

GROWTH FACTORS

Growth factors may restore the protective and reparative foundation of the colon, and therefore represent a possible therapeutic option for UC. Growth factors that have been identified as potentially beneficial in treating UC include transforming growth factor β (TGF-β), epidermal growth factor (EGF), keratinocyte growth factor-1 and 2 (KGF-1 or 2, also known as fibroblast growth factor 7 or 10). Repifermin is a truncated, purified KGF-2 expressed in *Escheria coli*, and induces the proliferation of intestinal and colonic mucosa and reduces intestinal ulcers and inflammation in animal models[255]. Intravenously administered repifermin (1-50 μg/kg) for five consecutive days did not yield different rates of clinical response or remission at wk 4 compared to placebo in patients with active UC[255]. Among other reasons, the authors suggested that under-dosing and/or under-powering could have accounted for the negative findings. EGF is a mitogenic peptide produced by salivary and duodenal Brunner's glands: topical application is beneficial in wound healing and systemic EGF is useful in treating neonatal necrotizing enterocolitis[256]. An 83% remission rate was demonstrated in patients with mild to moderate left-sided UC (n = 24) randomized to daily EGF enemas for 2 wk compared to 8% in the placebo group ($P < 0.001$); disease activity, endoscopic and histologic scores remained significantly better in the EGF group through 12 wk[256]. Rebamipide is an amino acid analog of 2-(1H)-quinolinone used to treat gastric ulcers in Japan. It aids mucosal healing by stimulating local prostaglandin synthesis and epithelial cell regeneration *via* upregulation of EGF and its receptor, neutrophil suppression, and decreased production of inflammatory cytokines stimulated by NSAIDs and/or *H pylori*[257]. A small open-label trial in which twice daily Rebamipide enemas were given to patients with UC proctitis for one month demonstrated significant clinical, endoscopic and histopathologic improvement[258]. Larger

controlled trials are needed to evaluate this class of therapy in UC.

CURCUMIN

Derived from tumeric, curcumin appears to inhibit NFκB and possesses anti-inflammatory, anti-microbial and tumor-suppressing characteristics; it has been shown to prevent and treat colitis in animal models. Fewer patients with quiescent UC (n = 82) randomized to 5-ASA plus curcumin compared to 5-ASA plus placebo relapsed over six months (2 *vs* 8, $P = 0.04$)[259].

NICOTINE

While CD is exacerbated and more difficult to treat in active smokers, UC by contrast is a disease of non-smokers. Older patients diagnosed with UC are commonly ex-smokers[260]. One study showed that while the risk of developing UC was not statistically different between those who never smoked and active smokers, ex-smokers were at greater risk to develop UC, suggesting that cessation of smoking increases risk[261]. It is speculated that nicotine alters systemic and/or gut immune function in a protective way; the exact mechanism remains unknown[262]. A Cochrane review found that transdermal nicotine (15-25 mg/d for 4-6 wk) induces remission more readily than placebo although benefit was not greater than mesalamine or corticosteroids. More patients experienced side effects (nausea and light-headedness) with nicotine compared to the other medications[263]. In contrast, transdermal nicotine is no more effective than placebo in the maintenance of remission[264], and nicotine enemas are no more effective than placebo in achieving remission in patients with distal UC[265].

APHERESIS

Selective apheresis of leukocytes, including the targeted removal of monocytes, granulocytes, and lymphocytes is a growing area of research in the treatment of UC. Review of leukocyte apheresis studies shows efficacy in inducing remission across various UC populations in small, open trials[266], but the inherent process of apheresis makes controlled studies difficult to conduct. Two larger trials have demonstrated that leukocyte apheresis (n = 76) and granulocyte/monocyte apheresis (Adacolumn®) (n = 69) are equally or more effective than steroids in the induction of remission[267,268], with fewer adverse events[267] and greater steroid-sparing effects[268]. In the only sham-controlled trial to date, 19 patients with moderate to severe UC treated with five weekly sessions of either leukocyte apheresis (followed by every other week for 4 wk) or sham apheresis demonstrated that the leukocyte apheresis group had significantly greater clinical improvement (80%) than the sham group (33%)[269]. Maintenance of remission after apheresis has been equivocal: in one study of 71 patients with active UC treated with leukocyte apheresis, only 27% of those with an initial response (n = 53) maintained remission for more than six months; rapid response to treatment was the only factor correlated with long-term response in multivariate analysis[270]. In another study, however, 26 of 33 patients maintained remission at one year after 11 weekly sessions of granulocyte/monocyte apheresis[271]. Apheresis may be effective in other settings as well, including a small group of patients with toxic megacolon[272], acute pouchitis[273] and a patient with pyoderma gangrenosum[274].

PROBIOTICS

While there is suggestion that probiotics may benefit patients with active UC, data are limited. Open-label studies with VSL #3[275] and *Saccharomyces boulardii*[276] have shown promise, while a small randomized controlled trial of bifidobacterium fermented milk (100 cc/d for 12 wk) in 20 patients demonstrated significant clinical, endoscopic and histologic improvement over placebo[277]. *Bifidobacterium longum* combined with Synergy, a prebiotic (inulin oligofructose growth substrate) showed a trend toward endoscopic improvement over placebo and significantly decreased inflammatory cytokines such as TNFα and IL-1 in the treated group[278]. No difference in relapse rates were seen among 327 patients with quiescent UC given *Escherichia coli* Nissl 1917 compared to mesalamine over 12 mo[279]. Among 187 patients with inactive UC given either *Lactobacillus* GG, mesalamine or a combination of the two treatments, no differences in relapse rates at six or twelve months were seen across the three groups, although treatment with lactobacillus GG alone or together with mesalamine prolonged relapse-free time[280]. A review by Rioux and Fedorak, showed that VSL #3 has also been beneficial in the maintenance of remission for pouchitis[148].

TRICHURIS SUIS

Although the mechanism is unclear, helminthic colonization has been theorized to be protective against the development of IBD based both on epidemiologic and animal model data. In a randomized controlled trial of 54 patients with active UC, those who received *Trichuris suis* ova every 2 wk for 12 wk had a greater response rate (43%) compared to those who received placebo (16.7%), $P = 0.04$[281]. While intriguing, this study has been questioned regarding whether the statistically significant decrease in activity index of the treated group represents a clinically significant difference[282].

CONCLUSION

The treatment of IBD is a burgeoning field: in particular, the introduction of infliximab, an anti-TNFα medication, almost a decade ago, has been the most significant addition to the spectrum of therapeutic options in IBD, which for many years was primarily limited to 5-ASAs, antibiotics, steroids and immunomodulators. Medications may be used either to induce or maintain remission. Choice of therapy depends largely on the severity of disease, and may also be influenced by such factors as disease location, side effects and adverse events, as well as cost. While there is much debate presently regarding "top-down" compared to the traditional "step-up" treatment a reversal of the

"therapeutic pyramid" awaits more data regarding short- and long-term efficacy, safety, and pharmacoeconomic data. Aminosalicylates remain the standard induction and maintenance therapies for UC but have a more equivocal role in CD. Despite a paucity of evidence, antibiotics are also commonly used in CD, especially with colonic and perianal disease. Budesonide is effective as a first-line agent for ileal and/or right colonic CD although maintenance benefits remain to be proven. Conventional steroids induce remission for both CD and UC but are reserved for patients with moderate-severe disease or for those who have failed more first-line therapy. Immunomodulators such as 6-MP and azathioprine, as well as methotrexate, are effective steroid-sparing and maintenance therapies. Cyclosporine or tacrolimus can be effective for severe or refractory UC. Anti-TNF agents have been effective for patients with moderate-severe UC and CD, independent of concomitant medications. Potential side effects, costs and immunogenicity remain issues relating to current and future biologic agents. Novel therapies continue to be explored as the immunopathophysiologic underpinnings of IBD continue to be elucidated and an ultimate etiopathogenesis remains undetermined.

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Cancer in inflammatory bowel disease

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Abstract

Patients with long-standing inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer (CRC). Many of the molecular alterations responsible for sporadic colorectal cancer, namely chromosomal instability, microsatellite instability, and hypermethylation, also play a role in colitis-associated colon carcinogenesis. Colon cancer risk in inflammatory bowel disease increases with longer duration of colitis, greater anatomic extent of colitis, the presence of primary sclerosing cholangitis, family history of CRC and degree of inflammation of the bowel. Chemoprevention includes aminosalicylates, ursodeoxycholic acid, and possibly folic acid and statins. To reduce CRC mortality in IBD, colonoscopic surveillance with random biopsies remains the major way to detect early mucosal dysplasia. When dysplasia is confirmed, proctocolectomy is considered for these patients. Patients with small intestinal Crohn's disease are at increased risk of small bowel adenocarcinoma. Ulcerative colitis patients with total proctocolectomy and ileal pouch anal-anastomosis have a rather low risk of dysplasia in the ileal pouch, but the anal transition zone should be monitored periodically. Other extra intestinal cancers, such as hepatobiliary and hematopoietic cancer, have shown variable incidence rates. New endoscopic and molecular screening approaches may further refine our current surveillance guidelines and our understanding of the natural history of dysplasia.

Key words: Colon cancer; Inflammatory bowel disease; Dysplasia; Chemoprevention; Colonoscopy; Genomic instability

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INTRODUCTION

The two forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic, relapsing inflammation of the intestines. First described in a report by Crohn and Rosenberg in 1925[1], colorectal cancer (CRC) in patients with IBD has long been recognized. Even years after their disease is controlled with medications, IBD patients still live with the fear of developing cancer. CRC is the most common site of cancer in IBD, although cancer in other organs can occur. Together with the hereditary syndromes of familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer, IBD is among the top three high-risk conditions for CRC. To date, our understanding of CRC pathobiology has come from studies of patients with UC more so than Crohn's colitis. This review will focus mainly on the problem of CRC, and then address other cancers such as small intestinal adenocarcinoma, cholangiocarcinoma, and hematologic malignancies.

COLORECTAL CANCER IN PATIENTS WITH IBD

Prevalence and Incidence of CRC

The exact magnitude of the risk of cancer has been difficult to quantify due to various biases and methodological errors in published studies. Early estimates of CRC complicating UC were based on crude percentages and all were from major medical institutions, predominantly tertiary referral centers. Studies from these centers often included a greater proportion of patients who had more severe disease and cancer had already complicated their colitis. These center-based studies often overestimate the risk. Later population-based studies tended to include more patients with limited disease or those who have undergone colectomy and may thereby underestimate the true risk. Based on a 2001 meta-analysis by Eaden *et al*, including 116 studies from around the world, the prevalence of CRC in patients with UC is approximately 3.7% overall and 5.4% for those with pancolitis[2]. In comparison, CRC in Crohn's disease has been less well studied. Early studies showed no statistically significant increase in cancer risk among the Crohn's disease patients. However, the lack of the risk of cancer in these studies was often due to inclusion of all patients with Crohn's disease and failed to correct for the small subsets of those with extensive, longstanding and unresected colonic disease. Hence, when patients with longstanding,

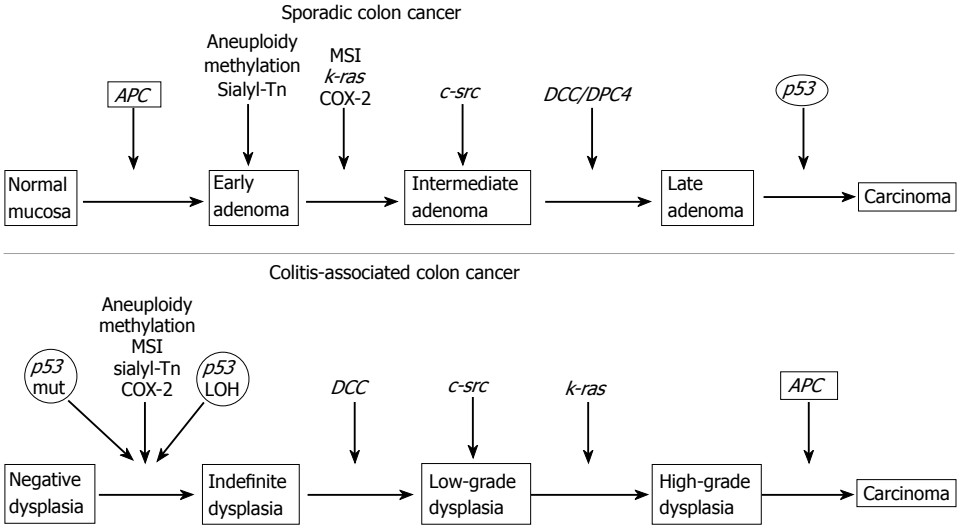

Figure 1 Comparison of molecular alterations in sporadic colon cancer and colitis-associated colon cancer. Mut, mutation. Modified from Ref 71. With permission.

anatomically substantial Crohn's colitis are considered, the risk of CRC is similar between Crohn's colitis and UC[3]. Indeed, a population-based study from Manitoba, Canada found that the risk for colon cancer among patients with both UC and Crohn's colitis is approximately 2-3 fold greater than the general population and that the risk of rectal cancer is increased 2-fold in UC but not Crohn's colitis[4].

Clinical features of colitis-associated CRC

Compared with sporadic colorectal carcinoma (SCC), CRC arising in patients with IBD has several distinguishing clinical features. Colitis-associated colorectal cancer (CAC) affects individuals at a younger age than the general population. CAC progresses to invasive adenocarcinoma from flat and nonpolypoid dysplasia more frequently than SCC. CACs more often have a higher proportion of mucinous and signet ring cell histology. There is background of chronic inflammation in colitis and a higher rate of two or more synchronous primary CRCs. The multifocality of CAC relates to the broader field effect of mucosal inflammation that gives rise to the neoplasia. In some studies, patients were found to have cancer more proximal in the colon. Finally, the sequence of molecular events leading from dysplasia to invasive adenocarcinoma is different from that of SCC (discussed below).

Molecular features of sporadic colon cancer

To place the molecular pathogenesis of colitis-associated neoplasia in proper perspective, it is important to appreciate the molecular events involved in the development of SCC. SCC arises as a result of genomic instability. The two main types of genomic instability that contribute to colon carcinogenesis are chromosomal instability (CIN) and microsatellite instability (MSI), accounting for 85% and 15% of SCC, respectively. Chromosomal instability results in abnormal segregation of chromosomes and abnormal DNA content (aneuploidy). As a result, loss of chromosomal material (loss of heterozygosity) often occurs, such as *APC* and *p53*. These genes can also be rendered nonfunctional by mutation.

Loss of *APC* function is typically an early event in SCC pathogenesis. The *APC* gene thus has been considered the "gatekeeper" of the colon (Figure 1). Some 85% of all sporadic and inherited colorectal tumors show loss of *APC* function, usually through protein truncation or allelic loss[5]. The *APC* gene is located on chromosome 5q21-q22. The key tumor suppressor function of the *APC* protein lies in its ability to destabilize free β-catenin[6]. Among the 15% of colon carcinomas that retain wild-type *APC*, point mutations have been found in β-catenin that change one of the four serine/threonine residues in the N-terminus, the putative targets of glycogen synthase kinase-3β (GSK-3β)[7]. These mutations thus render β-catenin refractory to phosphorylation by GSK-3β, increasing free β-catenin levels. Accumulation of stabilized free β-catenin is an early event and perhaps the initiating event in intestinal tumorigenesis. Inactivation of both *APC* alleles is found in a majority of small colorectal adenomas in humans and in the smallest detectable tumors in mice heterozygous for an inactivating mutation in *APC*[8]. Furthermore, intestine-specific expression of a dominant-negative form of β-catenin, which lacks the putative GSK-3β targets sites, leads to the development of adenomas[9]. How the loss of *APC* or stabilization of β-catenin leads to development of cancer is not yet fully understood. Once a sporadic adenoma forms, other changes in genetic regulation occur, such as induction of *k-ras* oncogene and loss of function of tumor suppressor genes on chromosome 18q in the region of the deletion in colon cancer (*DCC*) and in pancreatic cancer (*DPC*4) genes. Loss of *p53* gene function occurs late and is believed to be the defining event that drives the adenoma to carcinoma.

Tumors that arise *via* the CIN/tumor suppressor gene pathway are typically microsatellite stable (MSS). The remaining 15% of sporadic CRCs arise through the MSI pathway. The MSI pathway involves the primary loss of function of genes that usually repair DNA base-pair mismatches that occur during the normal process of DNA replication in dividing cells. In humans, at least six different proteins (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH6*, and *hMLH3*) comprise the mismatch repair system[10]. These proteins form specific heterodimers to coordinate DNA repair and the recruitment of other proteins, such

as polymerases and helicases, necessary for mismatch repair. Germline mutations of DNA mismatch repair genes, predominantly *hMLH1* (33%) and *hMSH2* (31%), are responsible for the familial syndrome of heriditary nonpolyposis colon cancer[11]. In addition, approximately 15% of sporadic tumors from the colon, rectum, and other organs demonstrate MSI[12]. Interestingly, the most common mechanism causing MSI in sporadic colon cancers is not genetic mutation, but rather transcriptional silencing of *hMLH1* as a consequence of methylation of the *hMLH1* promoter[13].

Epigenetic alterations can also contribute to altered gene expression in colon carcinogenesis. The CpG island methylator phenotype occurs when cytosines in the promoter region of genes become extensively methylated. A number of human cancer genes that contain hypermethylation of promoter CpG islands have been identified[14]. These include *hMLH*, $p16^{INK4a}$, and *E-cadherin*. The process of methylation is an area of intense investigation, and it is anticipated that this line of research should help to define further the molecular pathways involved in CRC in a variety of clinical settings.

Molecular features of CRC in IBD

The neoplastic transformation in IBD is thought to be similar to the adenoma-carcinoma sequence in sporadic CRC (Figure 1). However, unlike SCC, where dysplastic lesions arise in one or two focal areas of the colon, in colitic mucosa, it is not unusual for dysplasia or cancer to be multifocal, reflecting a broader "field effect". Many of the molecular alterations responsible for sporadic CRC development also play a role in colitis-associated colon carcinogenesis. The emerging evidence suggests that the two major pathways of CIN and MSI also apply to CACs and with roughly the same frequency (85% CIN, 15% MSI). Distinguishing features of CAC, however, are differences in the timing and frequency of these alterations (Figure 1). For example, *APC* loss of function, considered to be a very common early event in SCC, is much less frequent and usually occurs late in the colitis-associated dysplasia-carcinoma sequence. Conversely, *p53* mutations in sporadic neoplasia usually occur late in the adenoma-carcinoma sequence, whereas in patients with colitis, *p53* mutations occur early and are often detected in mucosa that is non-dysplastic or indefinite for dysplasia.

Methylation is assuming increasing importance as a mechanism contributing to the genetic alterations in CAC (Figure 1). Methylation of CpG islands in several genes seems to precede dysplasia and is more widespread throughout the mucosa of UC patients[15].

CRC risk factors

Several factors have been identified which either increase or decrease CRC risk in the setting of IBD (Table 1). With respect to increasing CRC risk, the most important factor, reproducibly found across many studies, is the duration of colitis. CRC is rarely encountered before 7 years of colitis. The pooled estimate of cumulative CRC incidence in UC in Eaden's meta-analysis was 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease[2]. This may overestimate the risk since many studies in the meta-analysis were from the pre-surveillance era. Thus, a recent study by Rutter *et al* showed lower cumulative incidence of CRC in patients with UC even at the referral-based St. Mark's Hospital in London: 2.5% after 20 years of disease, 7.6% after 30 years, and 10.8% after 40 years of follow-up[16].

Table 1 Factors associated with CRC risk

Factors that increase CRC risk
Longer duration of colitis
Greater extent of colonic involvement
Family history of colorectal cancer
Primary sclerosing cholangitis
Young age of IBD onset (some studies)
Backwash ileitis (possibly)
Severity of histologic inflammation
History of dysplasia
Factors that decrease CRC risk
Prophylactic total proctocolectomy
Regular doctor visits
Surveillance colonoscopy
Chemoprevention

Extent of colitis is an independent risk factor for the development of CRC. The more colonic surface that is involved with colitis, the greater the CRC risk. However, different criteria exist regarding classification of the extent of colitis. Most early reports of surveillance programs used barium enema results at diagnosis as the standard for defining disease extent. A population-based study of over 3000 UC patients in Sweden examined by barium enema demonstrated that patients with proctitis had a standardized incidence ratio (SIR) of 1.7 [95% confidence interval (CI) 0.8-3.2] compared with age-matched population controls without UC, whereas those with left-sided colitis had a SIR 2.8 (95% CI 1.6-4.4), and those with pancolitis had SIR 14.8 (95% CI 11.4 to 18.9)[17]. Endoscopic and histologic evidence of inflammation are valid alternative criteria particularly in high risk patients. Very few studies have correlated CRC risk with histologic extent of disease, even though microscopic evidence of colitis is arguably a better indicator of disease extent than either endoscopic or radiographic changes. Mathy *et al*[18] reviewed 30 colectomy specimens and showed that dysplasia and CRC can arise in areas of microscopic colitis that are proximal to areas of gross colitis, suggesting that indeed histologic changes, even without colonoscopic alterations, might better define disease extent for the purposes of cancer risk. Backwash ileitis, defined as pancolitis with superficial involvement of the terminal ileum, has been suggested as an additional increased risk of CRC[19], but this requires additional confirmation.

IBD patients with a family history of CRC have at least a two-fold higher risk of CRC[20-22]. Both UC and Crohn's disease patients with primary sclerosing cholangitis (PSC) are at particularly high risk for developing colorectal neoplasia[23]. In a population-based Swedish study, the cumulative incidence of CRC in UC patients with PSC was 33% at 20 years[24]. By comparing patients with UC with and without PSC, Shetty *et al*[25] reported an adjusted relative risk (RR) for dysplasia or cancer of 3.15 (95% CI, 1.37 to 7.27) in patients with PSC. Current practice

guidelines recommend all patients with PSC not previously known to have IBD should undergo a colonoscopy to determine their status. For those patients with IBD, screening and subsequent surveillance should begin at the diagnosis of PSC, and if the patient progresses to the point at which liver transplantation is necessary, prophylactic colectomy should be considered. Young age at onset of colitis has been reported to be an independent risk factor for CRC in some[2,17,26], but not all studies[27,28]. There is insufficient evidence to support starting screening and surveillance before 8 years of disease in these patients. The severity of colitis has not been considered an independent risk factor for CRC when activity of disease is defined according to the frequency of clinical exacerbations[22]. However, a case-control study by Rutter *et al* showed that increased severity of inflammation, both endoscopically and histologically, correlates with increased frequency of dysplasia[29]. It is important to realize that the patient with quiescent IBD is also at increased CRC risk.

With regard to reducing CRC risk, there are two main choices: removing the colon versus conducting a lifelong program of surveillance (Table 1). Although prophylactic total proctocolectomy after 7-10 years of colitis would prevent most cancers, it would result in many colectomies that were not necessary and a substantially altered quality of life for patients. Thus cancer prevention in this patient population has focused on periodic surveillance colonoscopies. Surveillance should be viewed as a program that includes regular visits to the doctor, the use of medications to control inflammation (some of which may have chemopreventive effects, see below), and regular colonoscopies. The goal of surveillance colonoscopy is to detect neoplastic lesions before they become biologically dangerous. Thus, the detection and interpretation of dysplasia is crucial to successful surveillance.

DIAGNOSING DYSPLASIA

Macroscopic classification of dysplasia

By definition, dysplasia is unequivocal neoplasia. Despite considerable heterogeneity in appearance[30], dysplasia in IBD is often classified macroscopically as raised or flat, depending on whether it corresponds to an endoscopically visible lesion. Raised lesions, conventionally referred to by the term DALM (dysplasia associated lesion or mass)[13], can appear as polyps, bumps, plaques and velvety patches[31,32]. Such lesions can blend easily with the gross inflammatory abnormalities commonly encountered in colons with IBD, making their endoscopic detection difficult even for experienced practitioners.

Unlike sporadic cancers arising from polypoid lesions, IBD-associated cancers can arise from flat dysplastic lesions. Flat dysplasia is detected microscopically in random biopsies from unremarkable mucosa. Its detection therefore depends on adequate sampling of the mucosa by the endoscopist, or more recently, by chromoendoscopy methods which highlight suspicious lesions and permit targeted biopsies (see below). If random biopsies are performed without dye spray enhancement, it has been estimated that to exclude dysplasia with a 90% certainty, 33 biopsy specimens are required, and to increase the accuracy to 95%, nearly twice the number of biopsy specimens are required[33]. Current surveillance strategies recommend annual colonoscopy with multiple biopsy specimens (4 circumferential) taken from every 10 cm of diseased colon, with additional biopsy specimens at sites of strictures or raised lesions[34]. However, questionnaire surveys have suggested that the number of biopsies taken by endoscopists in routine practice often falls short of recommended guidelines[35,36].

The significance of dysplasia in endoscopically visible lesions came from studies that reported high rates of cancer when patients with such lesions underwent colectomy[31,32]. Blackstone *et al* reported cancers in 7 of 12 DALM-bearing colons, including 5 with only mild or moderate dysplasia in the preoperative biopsies[31]. A subsequent compilation of published results from ten surveillance programs reported cancers in 17 of 40 (43%) colectomies performed because of a DALM[37]. It was concluded that DALM is an indication for colectomy irrespective of the grade of dysplasia in preoperative biopsies. While not fully appreciated at the time, the original studies of DALMs dealt exclusively with lesions that could not be removed endoscopically for microscopic examination. Thus, the significance of such lesions as an indication for surgery is similar to that of endoscopically non-resectable sporadic adenomatous polyps, which frequently harbor invasive cancer at the polyp base despite the presence of low-grade dysplasia in their more biopsy-accessible upper portions.

More recently, we have come to realize that not all types of polypoid dysplasia in patients with IBD carry the same significance. Some polyps may be adenomatous polyps unrelated to colitis and can be managed by endoscopic polypectomy like polyps in the general population[38-40]. One example is the dysplastic polyp encountered in a bowel segment that is entirely free of disease (e.g., in the proximal colon of a patient with left-sided ulcerative colitis). In such cases, one would take the precaution to biopsy the mucosa surrounding the polyp to assure the absence of microscopic disease. Similarly, a dysplastic polyp with a well-defined stalk can be regarded as a sporadic adenoma, even when encountered in a colitic region, if the mucosa lining its stalk is non-dysplastic.

Conservative management is also reasonable for dysplastic polyps that are "adenoma-like"[41,42]. These polyps are endoscopically indistinguishable from sporadic sessile adenomatous polyps, i.e., discrete and ovoid or round, are completely resectable by the endoscope, and are not surrounded by flat dysplasia. Such lesions have long posed a dilemma for endoscopists who were familiar with the DALM concept but reluctant to advocate colectomy for what appeared to be innocuous lesions and possibly nothing more than fortuitous adenomas. Histology has not provided a reliable means of making this distinction in individual cases[43], since the histological features of dysplasia in the setting of IBD and in true adenomas can be virtually identical. A 1999 study from The Mount Sinai Hospital in New York reported that conservative management of a cohort of 48 UC patients with a total of 70 such polyps, including 3 with high-grade dysplasia, resulted in no adverse outcomes during a mean follow-up

period of 4.1 years[41]. Similar conclusions were reached in a concurrent study from Brigham and Women's Hospital[42] that was confirmed upon longer follow-up[44]. As a result, the burden of deciding whether a polyp qualifies as adenoma-like rests with the endoscopist. Molecular markers may ultimately afford a more objective means of making these distinctions[45,46], but to date, these analyses are not applicable to routine clinical practice.

Microscopic classification of dysplasia

Gastrointestinal dysplasia is defined microscopically as replacement of the native intestinal epithelium by an unequivocally neoplastic, but noninvasive, epithelium[47]. It is synonymous with the term "intraepithelial neoplasia" used in other organ systems. A standardized classification system of dysplasia in IBD was established and divided dysplasia into five categories: negative for dysplasia, indefinite for dysplasia, low-grade dysplasia (LGD), high-grade dysplasia (HGD) or invasive cancer[47]. A further subdivision of the indefinite category includes probably negative, probably positive, unknown, however many pathologists regard this as optional.

The cellular abnormalities that define dysplasia in IBD are analogous to those characterizing neoplastic tissue in general, namely nuclear abnormalities reflecting inappropriate cellular proliferation and cytoplasmic abnormalities reflecting clonality and aberrant differentiation. The distinction between low- and high-grade dysplasia depends upon the distribution of nuclei within the cells, low-grade dysplasia being characterized by nuclei that remain confined to the basal half of the cells and high-grade by nuclei that are stratified haphazardly between the basal and apical halves[47]. Not surprisingly, pathologists are frequently confronted with biopsies that lie in a gray zone between the two categories, and some degree of subjectivity is therefore unavoidable. The diagnostic category "indefinite for dysplasia" is an acknowledgement of the difficulty pathologists face in discriminating between dysplasia and reactive epithelial changes, although experienced pathologists are usually able to discriminate between the two.

There is inconsistency among pathologists in the diagnosis of dysplasia on biopsy. In one study[48], there was only 60% agreement for a diagnosis of LGD. Similarly Lim *et al*[49] found that the kappa coefficient for interobserver agreement between ten pairings of five specialist gastrointestinal (GI) pathologists ranged from 0.06 to 0.39. Other studies comparing diagnoses of dysplasia among different pathologists, both prospectively and retrospectively, have concluded that levels of interobserver agreement are fair at best even among specialists in gastrointestinal pathology[50-54]. The best agreement levels tend to occur at the two extremes of negative and high-grade dysplasia and the poorest levels in the two gray zones between low-grade and high-grade dysplasia and on either side of indefinite for dysplasia. From a practical standpoint, it has been recommended that diagnoses carrying serious management implications be reviewed by at least one additional pathologist with expertise in this area[55].

BIOLOGY OF DYSPLASIA

Natural history of dysplasia

The natural history of dysplasia is a key factor contributing to the outcome and success of surveillance. The model shown in Figure 1 suggests that colitic mucosa progresses in a systematic fashion: no dysplasia, indefinite dysplasia, LGD, HGD, and finally invasive cancer. Although this is a useful paradigm that facilitates the study of cancer risk markers in IBD, it remains unclear whether dysplasia of one grade may "progress" (or "regress") to another grade. For example, patients undergoing regular colonoscopic surveillance have developed CRC without any prior dysplasia, and it is not necessary for LGD to progress to HGD before cancer arises in the colon[30,56]. This highlights the need to develop markers that are complementary to dysplasia for predicting CRC risk in IBD patients-a subject of ongoing investigation.

In the meantime, we currently rely upon the histological identification of dysplasia to make management decisions. Refinements in interpreting dysplasia based on the 1983 standardized histological criteria[47] have enabled a more accurate prediction of which patients are more likely to progress to advanced neoplasia by excluding those whose biopsies only show reactive changes secondary to inflammation. This was amply illustrated by the St. Marks group who found that the 5-year cumulative rate of progression from LGD to HGD or cancer rose from 16% to 54% once biopsies were more precisely reclassified[53,57].

Dysplasia (of any grade) is associated with a risk of concurrent CRC in IBD. An early study reported 12 cases, described as unresectable single polypoid masses, collections of polyps, or plaque-like lesions, 7 were found to have adenocarcinoma upon colectomies despite multiple biopsies that had not detected it[31]. In a review of ten prospective surveillance trials, 43% of patients who underwent colectomy because of DALM had coexistent CRC, 42% (10 of 24) patients with HGD, and 16% (3 of 19) patients with LGD who underwent immediate colectomy had synchronous CRC[37]. Ullman and colleagues at The New York Mount Sinai Hospital performed a retrospective cohort analysis of 46 patients with UC who had flat LGD but did not undergo immediate colectomy. They found that 27% (3 of 11) patients who underwent colectomy within 6 months of the initial detection of flat LGD had a surprise finding of cancer or HGD[56]. More recently, Rutter *et al* from the St. Mark's Hospital reported 20% of patients with LGD who proceeded to colectomy had concurrent adenocarcinoma and 39.1% who had follow-up of the LGD progressed to subsequent HGD or CRC[16].

Assuming that early colectomy is not performed, what is the subsequent rate of progression? In the case of patients with HGD, 32% were found to have CRC after some follow-up period[37]. For those with LGD, the probability of eventually progressing to HGD or CRC was 16%-29%[37]. Data from St. Mark's Hospital indicate that the 5-year cumulative probability of progressing from LGD to HGD or cancer is 54%[53]. Strikingly similar results were obtained from The Mount Sinai Hospital, with a 5-year progression rate of 53% among 46 patients

with initial flat LGD[56]. A recent follow-up analysis from the St. Marks' group indicates slightly lower, but still substantial rates of progression[16]. Likewise, a series of 18 patients with LGD followed at the Mayo Clinic demonstrated a 33% 5-year progression rate[58]. Despite these rather similar results from three different patient populations, some authors have reported a substantially lower rate of progression. Befrits *et al* followed 60 patients with flat LGD from the Karolinska Institute in Sweden and found that none developed cancer and only 2 cases of progression to HGD in DALMs over a mean follow-up period of 10 years[59]. In the series by Lim *et al* from Leeds in the U.K., they reported that only 3/29 (10%) patients with LGD progressed to HGD or cancer after 10 years[49]. It is worth noting that in the latter two studies, the designation of LGD included specimens that were interpreted prior to the 1983 consensus guidelines, so they might have included cases with indefinite dysplasia.

Because of uncertainty of flat LGD[56,60], these studies have failed to achieve consensus on proper management of flat LGD. Hence, competing options should be discussed with each patient. A patient confirmed to have multifocal flat LGD (2 or more biopsies with LGD from a single screening or surveillance examination) or repetitive flat LGD (2 or more examinations with at least a single focus of LGD), should be strongly encouraged to undergo prophylactic total proctocolectomy. Furthermore, even for patients with confirmed unifocal LGD (only 1 biopsy positive for LGD in a screening or surveillance examination) should also be offered the option of undergoing prophylactic proctocolectomy, since evidence indicates that a 5-year rate of progression to HGD or CRC in this patient group seems to be similar to that of multifocal LGD[56].

THE MANAGEMENT OF DYSPLASIA

Once a decision is made to place a patient under surveillance, it is recommended that the patient formally agree to enter such a program and is willing to comply. Patients must be made to understand the limitations of surveillance and accept the concept that despite their own cooperation, dysplasia and cancer can still arise even in the hands of skilled endoscopists and pathologists[53].

The best proof that surveillance colonoscopy effectively reduces CRC mortality would be a prospective, randomized, controlled trial in which patients with longstanding IBD would undergo colonoscopic surveillance whereas controls matched for a similar risk profile would not. However, due to ethical, financial and practical limitations, this type of study will likely never come to pass. We must therefore rely on retrospective studies for insights as to the efficacy of surveillance colonoscopy. In a retrospective study by Choi *et al*, patients with chronic UC who developed cancer were divided into those who had surveillance colonoscopy and those who did not. Patients undergoing surveillance colonoscopy were found to have less advanced Dukes' stage CRC than those who did not and correspondingly had an improved 5-year survival rate (77.2% *vs* 36.3%, $P = 0.026$)[61]. However, the best evidence that colonoscopy reduces mortality from CRC in UC comes from case-control studies. In one such study, Karlen and colleagues identified 2 of 40 patients with UC and 18 of 102 controls who had undergone at least one surveillance colonoscopy who died as a result of CRC (RR = 0.29), CRC mortality was reduced by as much as 78%, although this did not reach statistical significance[62]. Another study found a similar degree of protection[22]. In these studies, a protective effect was found for individuals who had even one or two surveillance exams. There is also good evidence from prospective, albeit uncontrolled, studies of surveillance colonoscopy that in general, patients who comply with surveillance have cancers detected at earlier stages compared to those who do not comply[53,63]. Of course, cancers will still arise even within a surveillance program, but in balance, the practice of surveillance is beneficial[64].

Although the gastroenterology community has put its faith in surveillance colonoscopy to prevent CRC in IBD patients, surveillance has its limitations. Previous studies have shown low rates of observer agreement for the histopathologic interpretation of biopsy specimens between general pathologists and GI pathologists, or even among expert GI pathologists[47,48]. Endoscopists fail to take a sufficient number of biopsies to exclude the presence of dysplasia or cancer[33]. Unlike the dysplastic sporadic adenoma which typically assumes a discrete, polypoid shape surrounded by normal mucosa, dysplasia in the colitic colon can be flat or polypoid and is often difficult to discern. This may be particularly troublesome in a colon that is replete with inflammatory pseudopolyps. Furthermore, there is poor understanding of dysplasia amongst trained gastroenterologists. A survey of practicing gastroenterologists and senior GI fellows in the U.S. found that only 19% of respondents correctly identified dysplasia as neoplastic change[35]. Patient drop-out or non-compliance with surveillance contributes importantly to CRC mortality in IBD[49,53] and this must be considered when embarking upon, or continuing, a course of surveillance in individual patients.

RECOMMENDED SURVEILLANCE STRATEGY

Despite the limitations of surveillance colonoscopy, dysplasia remains the best marker for managing cancer risk in IBD. After approximately 7-8 years of colitis, patients should undergo an "initial" surveillance colonoscopy to determine the extent of colitis and check for neoplasia[55]. The entire colon should be examined, with approximately 4 biopsies taken every 10 cm. Some experts suggest taking more biopsies in the distal rectosigmoid (e.g., approximately every 5 cm) since the distribution of neoplasia in UC still shows a distal predominance. Biopsies should be taken from flat mucosa, but if any raised or suspicious lesions are encountered, these should be removed if possible and processed in separate specimen containers (with additional biopsies taken near the base of the polyp). If a patient is experiencing moderate-severe colitis symptoms, one option is to control the inflammation medically prior to performing the examination in order to minimize difficulties with the histological interpretation of

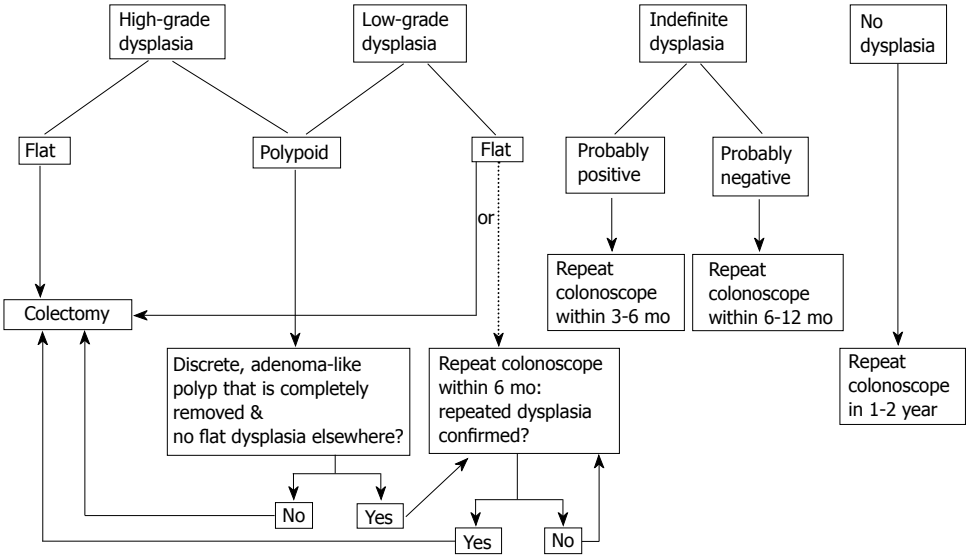

Figure 2 Suggested management scheme for dysplasia. Modified from Ref 71. With permission.

dysplasia. However, one should not defer the colonoscopy too long, since many expert pathologists now feel that they can readily interpret dysplasia even in the presence of active inflammation.

Figure 2 depicts a recommended surveillance strategy. If no dysplasia is detected, the examination should be repeated in 1-2 years. This interval derives in part from studies reporting that interval cancers can develop within 2 years after a surveillance examination[53]. If indefinite dysplasia is reported, the nature of the uncertainty should be discussed with the pathologist. If the suspicion of dysplasia is high (i.e., probably positive), repeat biopsy within 3-6 mo or less may be indicated; if low, the interval can be lengthened to every 6-12 mo. If LGD is detected in a discrete polyp that can be readily resected endoscopically and there is no flat dysplasia immediately adjacent to the polyp or elsewhere in the colon, surveillance can be continued, although the frequency of examinations can be temporarily reduced to every 3-6 mo, particularly to re-evaluate the area of polypectomy. Tattoo of the polypectomy site is advised to permit relocalization of the area on subsequent exams. If LGD is detected in flat mucosa (whether unifocal or multifocal), and is confirmed by a second expert GI pathologist, colectomy should be strongly considered. If the patient refuses, repeat surveillance exams should be undertaken within 3-6 mo or less. However, the patient should be advised that a negative subsequent examination is no assurance of safety, and that temporizing until there is histological progression to HGD or cancer as the indication for colectomy is risky. A patient in whom flat HGD or adenocarcinoma is found and confirmed by two expert pathologists should undergo colectomy unless serious co-morbidities dictate otherwise. If HGD is diagnosed in an adenoma-like polyp but it is completely removed without evidence of flat dysplasia in the adjacent mucosa or elsewhere in the colon, continued surveillance can be entertained. As with any set of recommendations, decisions should be individualized according to the situation of the patient. Hopefully, strategies for surveillance will become more refined as more knowledge of the natural history of dysplasia is obtained.

Patients who have only had small intestinal Crohn's disease without colonic involvement are not considered to be at high risk for CRC. For patients with Crohn's colitis, much less is known. To date, only one practice-based retrospective surveillance study has been reported in patients with Crohn's colitis[65]. Of 259 patients with Crohn's colitis affecting at least one-third of the colon for at least 8 years, 16% were found to have dysplasia or cancer over a 16 year period in which 663 examinations were performed, and there were no cancer deaths. While we await additional data on the subject, it seems wise to follow a UC-based surveillance strategy for patients with at least 8 years of substantial Crohn's colitis. An important question that remains to be clarified is whether patients with dysplasia or cancer in the setting of segmental Crohn's colitis can undergo segmental resection of the involved area or should proceed to a more extensive UC-like surgical approach. Another dilemma that is encountered with Crohn's colitis is the management of strictures. This occurs more so than with UC. Finding a stricture in a colon of a patient with UC usually means an underlying malignancy, especially if the stricture is causing symptoms, and is located in the proximal colon[66]. However, since most strictures in Crohn's colitis are benign, the patient can often be managed conservatively. Surveillance of such patients often requires using a narrower colonoscope or sometimes dilating a stricture to visualize the proximal mucosa[65]. Consideration should be given to adding brush cytology of strictures to regular forceps biopsies, and performing a barium enema to evaluate for colonic wall irregularity.

New endoscopic techniques and molecular screening approaches

Recent publications on chromoendoscopy have demonstrated a greater yield for dye-spray targeted biopsies compared with numerous non-targeted biopsies and thus enhance the endoscopic detection of dysplastic lesions in colitic colons. In a randomized trial by Kiesslich *et al*[67], intraepithelial neoplasia was more than three times as likely to be detected using chromoendoscopy compared with surveillance using nontargeted biopsies (32/84 compared

with 10/81; $P = 0.003$). Rutter *et al* detected no dysplasia in 2904 non-targeted biopsies in 100 patients, but in targeted biopsies, nine dysplastic lesions were detected, seven of which were visible only with dye spraying by using indigo carmine instead of methylene blue[68]. A recent report by Ochsenkuhn *et al* from Munich, Germany showed a low frequency of colorectal dysplasia in patients with long-standing IBD by fluorescence colonoscopy with 5-aminolevulinic acid[69].

Other approaches worth mentioning are to examine the biopsy tissue of patients with IBD for molecular alterations. The best tested of these are aneuploidy, mutations in p53 and ras, and glycosylation abnormalities, particularly increased expression of sialyl Tn antigen (sialyl 2,6 N-acetylgalactosamine)[70]. Because the DNA shed into stool should theoretically provide a more comprehensive sampling of abnormal cells than random pinch biopsies, stool DNA testing could potentially contribute to the management of patients with long-standing IBD who are at risk for developing CRC.

Chemoprevention

Despite the relative protection afforded by surveillance colonoscopies in IBD, there are still patients who develop CRC despite seemingly optimal surveillance. This raises the issue of whether chemoprevention in the form of either medications or dietary supplements might help reduce the risk of CRC in IBD[71].

Aspirin and other NSAIDs markedly reduce the incidence of, and mortality from, sporadic CRC. Since many patients with IBD take NSAIDs in the form of 5-aminosalicylates (5-ASA), investigators have asked whether 5-ASA compounds might also be protective. Although no study to date has been performed in a prospective manner specifically to address this question, the available data suggest that this may be so[72]. If 5-ASA compounds prevent colonic neoplasia by suppressing inflammation, it follows that other anti-inflammatory medications used in IBD patients should also be protective against CRC. Although one study reported that the use of systemic steroids, and even topical steroids resulted in a significant CRC risk reduction[22], and others confirm this observation[73], steroids cannot be used long-term for chemoprevention. There appears to be no chemopreventive activity of 6-mercaptopurine or azathioprine[74].

In the setting of sporadic CRC, low folate intake has been associated with an increased risk for developing colorectal adenomas and carcinomas[75,76]. Patients with chronic IBD are predisposed towards folate deficiency because of inadequate nutritional intake, excessive intestinal losses with active disease, and reduced intestinal absorption from competitive inhibition from sulfasalazine use. Results of two studies suggest a trend towards protection against CRC in folate users, although neither study demonstrated statistical significance[77,78]. Nonetheless, since it is rather safe and inexpensive, folate supplementation should be considered for CRC risk reduction in patients with longstanding IBD.

In animal models of colon carcinogenesis, ursodiol inhibits carcinogenesis-an effect that may be due to the ursodiol reducing the colonic concentration of the secondary bile acid deoxycholic acid. Ursodiol also has anti-oxidant activity. A study of UC patients with PSC demonstrated that ursodiol use was strongly associated with decreased prevalence of colonic dysplasia[79]. This protective effect remained after adjusting for duration of colitis, age at onset of colitis, and sulfasalazine use. In a follow-up to the randomized, placebo-controlled trial by Pardi *et al* at the Mayo Clinic, 52 patients with chronic PSC and chronic UC (mean 13 years) were followed for a total of 335 person-years. Ursodiol use was associated with a significant protection against the development of dysplasia and cancer (RR = 0.26, $P = 0.034$)[80]. At the present time, however, we do not know whether ursodiol can prevent neoplastic progression in UC patients without PSC.

Recently, there has been interest in the role statins may play as chemopreventive agents in a variety of cancers. In a population-based case-control study of patients who had diagnosis of CRC in northern Israel between 1998-2004, statin therapy was associated with a modest reduction in CRC in the non-IBD population, but a substantial 94% risk reduction in patients with IBD was observed in a subset analysis of a small number of patients[81]. Further studies will need to verify this benefit.

Although a single retrospective cohort study suggests that therapy with 6-mercaptopurine is not chemopreventive (or carcinogenic), there remain insufficient data regarding the chemopreventive role of immunomodulators in order to make recommendations and likewise, whether patients who require immunomodulator therapy should continue their 5-ASA therapies[74].

Small bowel cancer in IBD

Most cancers of the small bowel in Crohn's disease are adenocarcinoma, usually in the terminal ileum or jejunum. The most common clinical presentation of small bowel cancer is intestinal obstruction[82]. Other important symptoms include diarrhea, weight loss, and abdominal fistulae. These symptoms are also found in Crohn's disease. Risk factors for developing carcinoma in small bowel segments of involved mucosa in patients with Crohn's disease are poorly defined, although case reports document them in strictured mucosa and fistulae[83-85]. Surgery should be considered if the fistulae or stricture cannot be adequately examined, or symptoms substantially worsen. There is some suggestion that 5-ASA compounds might lower the risk of small intestinal adenocarcinoma[86].

A number of studies have demonstrated an increased risk of developing adenocarcinoma of the small intestine with small intestinal Crohn's disease. Although the absolute number of cases of small bowel adenocarcinoma is low, because of the rarity of this cancer in the general population, the risk is approximately 10-12-fold greater than the general population[4,86]. In the Uppsala study[87], the investigators reported only one observed case compared with 0.3 expected cases, but the confidence interval was wide. In the Copenhagen study[88], two cases were observed *vs* 0.04 expected cases, a 50-fold increased occurrence. In the Tel Aviv study[89], none of the patients developed small cancer. The material was probably far too small to expect any small bowel cancer cases. In Oxford, a 10-fold increased relative risk was observed for cancer of the small

intestine[90]. A population-based Swedish study revealed a significantly increased number of cancer of the small intestine (standardized morbidity ratio, 15.64; 95% CI, 4.26-40.06), however, the occurrence of colorectal cancer was not increased[91]. Another population-based Canadian study encompassing the years 1984-1997 demonstrated an increased incidence rate ratio of carcinoma of the small intestine (17.4; 95% CI, 4.16-72.9)[4].

Patients with UC who have undergone total proctocolectomy with ileal pouch anal-anastomosis (IPAA) have a very small risk of dysplasia arising within the ileal mucosa of the pouch itself. The risk is thought to be higher in patients with chronic pouchitis and associated severe villous atrophy[92], but this has not been shown in all series[93]. Indeed, a study of 160 patients who underwent biopsy a total of 222 times with an average surveillance time of 8.4 years after surgery showed that in 1800 pouch-years of surveillance, only one patient had focal LGD of the pouch[94]. The risk of neoplasia is greater in the anal transitional mucosa between the pouch and the anal canal, particularly if a cuff of rectal mucosa has been left, and if the indication for the IPAA was rectal dysplasia or cancer[95]. While there are currently no guidelines for endoscopic surveillance after an IPAA procedure, in those patients who have chronic pouchitis and severe villous atrophy or whose original indication for IPAA was dysplasia or cancer, a program of periodic endoscopy with biopsies, paying particular attention to any anal transition zone, is reasonable.

Other cancers in patients with IBD

Squamous cell carcinoma of the anus has been reported in patients with longstanding, complicated perianal Crohn's disease[85]. Worsening perianal symptoms in such patients should warrant heightened vigilance for this tumor which often requires examination under anesthesia for adequate tissue diagnosis.

An increased risk for hepatobiliary cancers in patients with UC has been found in several[4,96-98] but not all[87] studies. For many of these patients, primary sclerosing cholangitis was the predisposing factor.

The risk of hematopoietic cancer in patients with IBD has been a growing concern. Early case series from The Cleveland Clinic[96] and The Mount Sinai Hospital[99], as well as other centers[100] reported an increase in leukemia in patients with UC. A recent large cohort study from Sweden[101], which included nearly 50 000 IBD patients concluded this population has a marginally increased risk of hematopoietic cancer, and in UC, lymphoma occurred as expected (SIR 1.0) but myeloid leukemia occurred significantly more often than expected (SIR 1.8). In Crohn's disease, there was a borderline significant increased lymphoma risk (SIR 1.3), essentially confined to the first years of follow up. However, population-based studies from Denmark[97], Sweden[87] and Canada[4] have failed to substantiate any increased risk of leukemia. Likewise, although an increased number of lymphomas have been reported in some case series[99,102], other series[96] and several population-based studies[87,91,97,98] do not support the notion that patients with UC or Crohn's disease are at increased risk of lymphoma. However, a population-based study from Canada reported an increased rate of lymphoma among male patients with Crohn's disease[4].

The risk of lymphoma or leukemia in IBD has raised concerns regarding the lymphogenic potential of immunomodulatory therapy. Following the introduction of tumor necrosis factor inhibitors in the treatment of Crohn's disease, subsequent reports indicated an excess of malignant lymphoma among treated patients[103,104] and raised fears of an iatrogenic lymphoma risk. However, these reports have also highlighted the lack of robust data on the expected occurrence of malignant lymphomas in TNF naïve (but otherwise treated) patients with IBD[105-107]. Studies examining the risk of lymphoma associated with azathioprine (AZA) and 6-mercaptopurine (6-MP) have yielded variable results. Heterogeneity in the type, dose, and duration of immunomodulatory therapy may be responsible for this discrepancy. A few studies with suboptimal dosing failed to demonstrate an increased risk of lymphoma[4,108-112]. In contrast to these reports, other studies have demonstrated an increased risk of lymphoma after purine analog therapy[113-115]. In one such study, Kandiel *et al* reported a 4-fold-higher risk of lymphoma inpatients treated with AZA or 6-MP compared with the general population[114]. Another recent study showed a statistically significant increase in the development of malignancies among IBD patients treated with 6-MP who developed sustained leucopenia[115].

FUTURE DIRECTIONS

The future looks promising with respect to new developments in the management of cancer risk in IBD. Chromoendoscopy is likely to be used more for management, but whether the predictable increase in the yield of dysplasia will alter the overall natural history remains to be studied. In the modern era of molecular diagnostics, tissue and even stool samples of patients with IBD can be investigated for molecular alterations. For example, University of Washington investigators have demonstrated that because there is often widespread genomic instability throughout the colon of IBD patients, it may be possible to analyze rectal biopsies by DNA fingerprinting or fluorescence *in situ* hybridization methods[116] to identify patients at particularly high risk. The advent of technology to extract human DNA from stool and look for specific DNA mutations associated with sporadic colon carcinogenesis[117,118] implies that a similar approach may also be worthwhile in IBD patients. It is anticipated that refinements in our knowledge of cancer biology, clinical practice, and molecular discovery will bring a new level of sophistication to the management of patients with longstanding IBD and lower the incidence of CRC in this high-risk population.

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TOPIC HIGHLIGHT

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Immunopathogenesis of inflammatory bowel disease

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Abstract

Crohn's disease and ulcerative colitis are chronic relapsing immune mediated disorders that results from an aberrant response to gut luminal antigen in genetically susceptible host. The adaptive immune response that is then triggered was widely considered to be a T-helper-1 mediated condition in Crohn's disease and T-helper-2 mediated condition in ulcerative colitis. Recent studies in animal models, genome wide association, and basic science has provided important insights in in the immunopathogenesis of inflammatory bowel disease, one of which was the characterization of the interleukin-23/Th-17 axis.

Key words: Crohn's disease; Ulcerative colitis; Innate and adaptive immune system

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INTRODUCTION

Inflammatory bowel disease (IBD) results from a complex series of interactions between susceptibility genes, the environment, and the immune system. The clinical features of the disease, histopathological findings, and the therapeutic efficacy of immunosuppressive drugs indicate an involvement of the immune system in the pathogenesis of the disease. Various components of the mucosal immune system are implicated in the pathogenesis of IBD. These components include luminal antigens, intestinal epithelial cells (IECs), cells of the innate and adaptive immune system, and their secreted mediators. An overview of the working hypothesis of IBD is depicted in Figure 1. Either a mucosal susceptibility or defect in sampling of gut luminal antigen leads to activation of innate immune response, possibly mediated by enhanced Toll-like receptor (TLR) activity. The stimulated dendritic cells (DC) then mediate the differentiation of naïve T-cells into effector T-cells. Crohn's disease (CD) is a predominately Th1 and Th17 mediated process, while ulcerative colitis (UC) appears to be predominately mediated through Th2 and NK T-cells. In this review, the effects of these components in the immunopathogenesis of IBD will be discussed.

BACTERIA AND THEIR ASSOCIATED MOLECULES IN THE INTESTINE

The distal ileum and colon contain high concentrations of bacteria (> 10^{12} organisms/g). These may include pathogens that could be directly responsible for initiating and promoting IBD in the context of an underlying genetic mucosal or immune defect. Studies have shown that there are differences in the microbiota between healthy and IBD subjects (Table 1). One of the differences is that there is a decrease biodiversity in IBD compared to healthy subjects by 30%-50%[1]. The reduction in diversity in inflammatory bowel disease was due to loss of normal anaerobic bacteria such as *Bacteroides*, *Eubacterium*, and *Lactobacillus* species[1]. Diversity is generally thought to be desirable by conferring resiliency to an ecosystem[2]. For example diversity would provide functional redundancy in the microbial community to ensure that key processes such as breaking down nutrients and preventing random chaotic fluctuation of bacterial subpopulations. Restriction of biodiversity in the human gut may lead to dysbiosis and result in mucosal insult. A second difference is that there are fewer *Firmicutes* in IBD compared to healthy subjects; 13 distinct *Firmicutes* ribotypes were identified in CD microbiota compared to 43 in healthy microbiota ($P < 0.025$)[3]. *Firmicutes* are Gram-positive class of bacteria that include the genus *Clostridium* and *Bacillus*. Third, there are pathogens that are found in increasing frequency in IBD and have been implicated to associate with its development. These pathogens include *Pectinatus*, *Sutterella*, *Fusobacterium*, *Verrucomicrobium*, various *Clostrodia*, *Mycobacterium paratuberculosis*, *M. paramyxovirus*, Listeria monocytogenes, and Helicobacter hapaticus[4,5]. Despite the differences in the microbiota, one must keep in mind that the dysbiosis seen in IBD patients may not be causal, but simply reflect the different ecological conditions of the inflamed gut such as changes in pH,

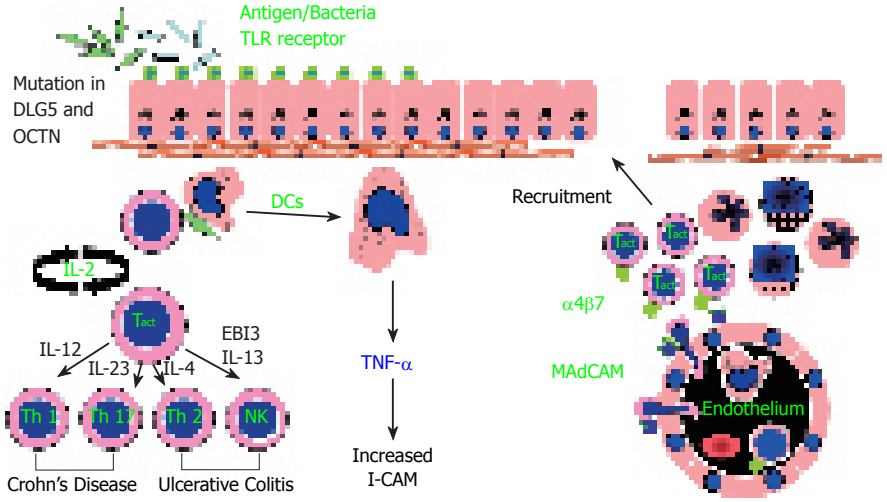

Figure 1 Working hypothesis of inflammatory bowel disease. A defect occurs in sampling of gut luminal antigen, possibly mediated by enhanced Toll-like receptor activity and controlled by other genetic factors (mutations in DLG5 and OCTN). Over-response to antigens results in stimulated dendritic cells (DC) that recruits and generates various T-cell subtypes, which then initiate a cascade of immunologic events leading to mucosal inflammation. Adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM1) are important for circulating mononuclear and polymorphonuclear cells to adhere and migrate to the inflamed gut mucosa. Crohn's disease (CD) is a predominately Th1 and Th17 mediated process, while ulcerative colitis (UC) appears to be predominately mediated through Th2 and NK T-cells.

Table 1 Comparison of microbiota between healthy and IBD subjects

Healthy subjects	IBD subjects
High biodiversity	Low biodiversity
Stable microbiota	Dysbiosis
Increased gut commensals	Increased gut pathogens
Higher firmicutes	Lower firmicutes

redox potential, substrate availability, *etc.*

The importance of the microflora in the induction of and maintenance of disease has been demonstrated in murine model of colitis. For example, mice that are deficient in the cytokines IL-2 or IL-10 or rats containing the *HLA-B27* transgene develop IBD in the presence of a normal microflora but not in a sterile germ-free environment[6-8]. Moreover, experimental colitis is attenuated when animals are treated with broad spectrum antibiotics[9]. Different bacteria may lead to different types of colitis in the same genetic host. In *IL-10*$^{-/-}$ mice, *E. coli* induced proximal colitis whereas *Enterococcus faecalis* lead to distal colitis[10]. Indirect evidence suggesting a role of the intestinal microbiota in IBD came from a double blinded, randomized controlled trials that showed imidazole antibiotics decrease the risk of post-operative recurrence of CD[11].

The surfaces of microorganisms typically bear repeating pattern of molecular structures as do their nucleotides. These motifs are designated as Pathogen Associated Molecular Patterns (PAMP). Some of the PAMPs include complex macromolecules such as lipopolysaccharide (LPS), peptidoglycan (PGN), polypeptides (flagellin), and nucleic acid (CpG rich DNA). Receptors of the PAMPs are called pattern recognition receptors (PRR). Toll like receptors (TLR) are examples of PRR. TLR recognition usually triggers the innate immune system, leading to an inflammatory response. Interaction between PAMPs and PRR are illustrated by the CpG dinucleotides activation of innate immunity *via* TLR9 (Figure 2). Oligonucleotides containing CpG motifs (CpG-ODN) prevented lesions and reduced the release of inflammatory cytokines when given before the DSS-induced colitis[12-14]. However, if CpG-ODN is given after DSS-colitis induction, the colitis is worsened[13-15]. In TLR9-deficient mice, the onset of DSS-induced colitis was not prevented, but chronic inflammation was reduced[12,15]. This is due to the dual anti-inflammatory effect of TLR9 signaling that is mediated through type I interferon (IFN)[16].

The study of autoantibody has provided evidence that immunologic responses to bacterial products is involved in the induction of inflammatory bowel disease. Reactivity to bacterial antigens was initially shown by findings of modest increase in serum antibodies to 7 bacterial pathogens in a group of CD patients[17]. Subsequently, several studies showed serum responses to various bacterial antigens and loss of tolerance to pathogenic as well as commensal bacteria in clones derived from peripheral and lamina propria T-cells from CD subjects[18-22]. This indicates that disordered features of T-cell microbial recognition and effector function are likely to be important to IBD disease biology.

Antibodies to specific bacterial antigens are clustered into 4 major groups in IBD patients: (1) antibody responses against oligomannan [anti-Saccharomyces cerevisiae (ASCA)][23]; (2) antibody responses to both *Escherichia coli* outer membrane protein C (anti-OmpC) and a CD-related protein from *Pseudomonas fluorescens* (anti-CD-related bacterial sequence)[24,25]; (3) antibody response to nuclear antigens [perinuclear antinutrophil cytoplasmic antibody (pANCA)][26]; or (4) antibody response to the flagellin, CBir1[27]. These antibodies provides immunophenotypic associations to distinguish between UC and CD. CD patients with high levels of IgG and IgA ASCA and the absence of pANCA have more aggressive, small bowel fistulizing and fibrosing disease, and patients with high-level pANCA, in the absence of ASCA, have an ulcerative colitis-like colonic disease[28-30]. In a prospective study, pANCA expression is associated with development of pouchitis after ileal pouch-anal anastomosis (IPAA)[30]. The site of pANCA production has been localized to the gastrointestinal mucosa and was not found in detectable amounts in the periphery, suggesting the importance of mucosal immune response to luminal antigen[31]. In fact, absorption of either human or mouse pANCA-positive sera with enteric bacterial antigens greatly reduced or abolished the specific perinuclear staining of pANCA,

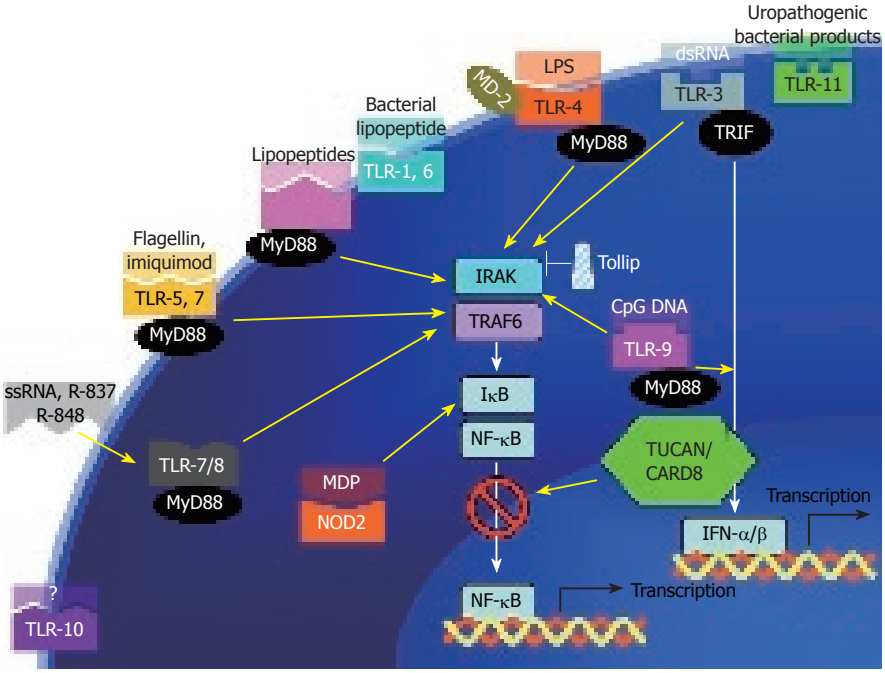

Figure 2 PRRs and their corresponding ligands. Toll-like receptors on the cell membrane (TLR-1, -6, -10, and -11) and intracellular (TLR-7, -8, and -9) selectively bind to various bacterial, viral, or fungal components. A major convergent pathway is through myeloid differentiation primary response protein MyD88, which activates NF-κB. The death domain of MyD88 then recruits downstream IL-1 receptor-associated kinase (IRAK) to the receptor complex. IRAK is then autophosphorylated and in turn recruits TNF receptor-associated factor 6 (TRAF6). TRAF6 then activates kinases including NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1). Inhibitor of NF-κB degradation (IκB) is subsequently phosphorylated and degraded, resulting in NF-κB nuclear translocation. NF-κB then activate genes involved in inflammatory response including IL-1b, TNF, IL-6, IL-8, and ICAM1. Toll-inhibitory protein (Tollip) is one of the negative regulators of the innate immunity. Activation of type I IFN (IFN-α/β) also has anti-inflammatory function in colitis.

indicating that pANCA seroreactivity reflects a cross-reactivity to enteric bacterial antigens[32].

The flagellin, CBir1, has been identified as an immunodominant antigen that is capable of inducing severe colitis in mice and eliciting antibody responses in approximately 50% of CD patients[33]. In contrast, patients with UC or other inflammatory gastrointestinal diseases and control subjects have little or no reactivity to CBir1[33]. Serum responses to CBir1 independently identify a subset of patients with small-bowel, internal-penetrating, and fibrostenosing CD that have no correlation to levels of ASCA, OmpC, and pANCA[23]. Molecular studies in CBir have also shed light on the underlying immunopathophysiologic mechanisms related to this antigenic response. CBir involves both the innate and the adaptive immune system. CBir flagellin is found to be the ligand for TLR5 (Figure 2)[34]. Bacterial flagellin is a structural protein that makes up flagella, which functions in bacterial chemotaxis and adhesion of host tissues. TLR5 is expressed on the basolateral surface of intestinal epithelial cells and hypothesized to be important in the recognition of invasive flagellated bacteria at the mucosal surface. TLR5-flagellin interaction leads to the activation of the transcription factor NF-κB, resulting in the transcriptional induction of proinflammatory cytokines and maturation of human dendritic cells (Figure 1)[35,36]. The expression of antibodies to CBir1 and transferring of a CBir1-specific CD4 + Th1 cells to induce colitis in mice is indicative of an adaptive immune response to this antigen[27].

These results show that some endogenous micro-organisms provide antigenic stimulation through PAMP activation of PRR to maintain intestinal homeostasis: either inflammation through the release of inflammatory cytokines or mucosal protection through type I IFN. In addition, serum reactivity to bacteria and their associated molecules may indicate a predisposition to certain mucosal antigen that could reflect the clinical manifestation and type of IBD. These distinct antibody response patterns to either UC or CD indicate unique pathophysiologic mechanisms to IBD.

INTESTINAL EPITHELIAL CELLS

The 400-mm^2 single layer of gut epithelial cells is the primary cellular barrier to prevent antigens from encountering the immune system. The gut maintains an extensive and active gut-associated lymphoid tissue (GALT). Peyer's patches (PP) are aggregates of lymphoid tissue that are interspersed at intervals just beneath the gut epithelium. They are comprised of B-cell follicles located under specialized areas, or 'domes,' of epithelium known as follicular-associated epithelium (FAE), with T-cell zones occupy the areas between follicles[37]. FAE contains cells called multi-fenestrated or M cells whose function is to transport luminal antigen into the dome area of the follicle[37]. Dendritic cells (DCs) are antigen presenting cells that act as sentinels, sending processes between gut epithelial cells and sample commensal and pathogenic bacteria[38]. Defects in the epithelial barrier may allow GALT to be exposed to excess or harmful luminal antigens, resulting in the production of pro-inflammatory cytokines such as TNF-α and mucosal inflammation.

Tight junction between epithelial cells allows for the selective entry of fluids, nutrients, and micro-organisms. Normal gut permeability is dependent on an intact epithelium, surface mucus, peristalsis, and the secretion of host protective factors. Alterations in gut permeability factors have been reported. Two genes that are associated with Crohn's disease which affect mucosal permeability are the IBD 5 gene organic cation transporter (*OCTN*) and guanylate kinase *DLG5* (Figure 1). Missense mutations in the *OCTN* gene form a haplotype that is associated with susceptibility to Crohn's disease and the mutant protein has impaired ability to pump xenobotics and amino acids across cell membrane[39]. The other protein, *DLG5*, is a scaffold protein involved in the maintenance of

epithelial integrity. Two distinct haplotypes in the *DLG5* gene represented by nonsynonymous single-nucleotide polymorphism in the gene are associated with IBD[40]. It is proposed that the mutation probably impedes scaffolding of *DLG5* and thus, affect epithelial polarity[40]. Altered mucus production has also been found in IBD patients with thinner than normal colonic mucus layer in UC and a thicker than normal layer in CD[41]. Several studies have shown that the there is an increased number of adherent bacteria in IBD patients[42-44]. This relationship holds true in both the mucus layer and at the epithelial surface where bacteria are less abundant. It is possible that the disruption of the function of *OCTN* and *DLG5* and abnormal mucus composition alters the gut permeability. Altered gut permeability may lead to increased bacteria adherence and inappropriate exposure of the mucosal immune system to bacterial products causing inflammation.

INNATE IMMUNITY

Studies described above indicate that microbial structures can cause gut mucosal inflammation by involving the innate immune system. Adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM1) are important for circulating mononuclear and polymorphonuclear cells to adhere and migrate to the inflamed gut mucosa (Figure 1)[45]. Proinflammatory molecules are preferentially produced by innate immune cells that have migrated to the inflammatory mucosa owing to increased expression of PRR such as TLR[46]. Antigen-presenting cells such as dendritic cells (DCs) and macrophages possess TLRs with different specificities for microbial products and induce innate immune response. Immunopathogenic role of TLR signaling in IBD is in the process of being elucidated.

NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN (NOD) PROTEINS

NOD proteins are a distinct subset of PRRs that have important roles in innate immunity as cytoplasmic sensors of microbial components, allowing for regulation of inflammatory processes and apoptosis[47]. The importance of these PRRs is highlighted by the fact that mutations in the gene that encodes NOD2 occur in a subset of patients with CD and polymorphisms in the gene that encodes NOD1 are associated with IBD[48-50]. The NOD1 protein (encoded by caspase-recruitment domain 4 gene, *CARD4*) and NOD2 protein (encoded by CARD15) are expressed by antigen-presenting cells (APCs) and epithelial cells[47]. NOD2 is a general sensor of most bacteria because it recognizes muramyl dipeptide (MDP), which is a component of both Gram-positive and Gram-negative bacteria[51]. In contrast, NOD1 senses mostly Gram-negative bacteria because the ligand for NOD1, peptidoglycan (PGN) derived peptide γ-D-glutamyl-mesodiamino-pimelic acid (iE-DAP), is not present in Gram-positive bacteria except for *Listeria* and *Bacillus spp*[52]. The main outcomes of NOD1 and NOD2 activation is the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathway (Figure 2)[51,53-55]. In addition, activation of NOD1 by its ligands leads to the activation of JUN N-terminal kinase (JNK)[47]. Activations of NF-κB, MAPK, and JNK pathways lead to the production of pro-inflammatory mediators[47].

There are 3 models of how *CARD15* mutations are associated with CD, however, studies have shown inconsistencies in these models. The first view is based on the study that showed NOD2 is a negative regulator of IL-12 mediated by PGN[53]. Loss-of-function mutation in CARD15 results in an excessive NF-κB dependent IL-12 response by splenic macrophages to create a milieu that supports Th1 cell mediated colitis. This is supported by findings that production of IL-12p70 by peripheral blood monocytes derived dendritic cells in response to PGN is higher in patients with CARD15 mutation than in normal subjects[56]. In contrast to the study by Watanabe *et al*, another study that used bone-marrow derived macrophages did not result in enhanced proinflammatory cytokine production[55]. Other studies show that CARD15 mutation results in reduced anti-inflammatory cytokine responses (IL-10 and TGF-β) to several TLR ligands[57,58]. These indicate that NOD2 signaling should augment an anti-inflammatory response and not inhibition of TLR responses in normal individuals. However, defective anti-inflammatory response secondary to CARD15 mutation does not sufficient explain increased cytokine production that is characteristic of CD.

The second model suggested that a loss-of-function NOD2 lead to susceptibility to CD as a consequence of impaired host defense. This may be due to impaired NOD2-dependent α-defensin production by Paneth cells, and thus, lead to impaired control of pathogenic bacteria and inflammatory condition[59]. This is also supported by the finding that CD patients with CARD15 mutation have a more pronounced defective production of a-defensin-5 and -6[60]. Consistent with the impaired mucosal host defense, NOD2 deficient mice developed worse liver infection to *L. monocytogenes* when the pathogen was given orally compared with systemic administration[55]. However, there are several studies that did not support the idea that CD is due to impaired host defense as a consequence of NOD2 mutation. One of the strongest argument is the data that mice in with ablation Paneth-cell population or mice that lacks α-defensins develop evidence of acute or chronic inflammation[61,62]. In addition, NOD2-deficient mice that were administered *L. monocytogenes* did not cause mucosal infection[55].

The third model proposes gain-of-function mutations in the *CARD15* gene that are associated with CD. This possibility is supported by studies in mice carrying a mutation that is analogous to the most common CD susceptibility allele, 3020insC[63]. The mutant mice show increased NF-κB activation and enhanced production of IL-1β upon MDP stimulation of bone marrow-derived macrophages[63]. Inconsistent with the above study, peripheral-blood mononuclear cells isolated from CD patients that carrie the 3020insC allele show a defect in IL-1β secretion[64]. Additionally, epithelial cells transfected with 3020insC CARD15 mutation have defective NF-κB activation upon MDP stimulation[51,65].

There are several views and contradictory findings on the role of NOD protein in the pathogenesis of IBD.

Among the unanswered questions are whether CARD15 mutations confer a gain- or loss-of protein function. Perhaps the mutations have different effects in different context, i.e. in the APC *versus* epithelial cells or in the mucosa *versus* the periphery. More studies are needed to elucidate the immunopathogenesis of CARD15 mutations in IBD.

TOLL LIKE RECEPTOR AND ACTIVATION OF INNATE IMMUNE RESPONSE

Eleven TLRs have been identified which are characterized by three common structural features: (1) divergent ligand binding extracellular domain with leucine rich repeats; (2) short transmembrane region; (3) a highly homologous cytoplasmic Toll/interleukin 1 receptor (TIR) domain. Even though each type of TLR engages a different PAMPs, a major convergent pathway is through myeloid differentiation primary response protein MyD88, which activates NF-κB. Upon activation, TLRs form homodimers leading to a conformation change in the cytoplasmic TIR domain and recruitment of MyD88[66]. The death domain of MyD88 then recruits downstream IL-1 receptor-associated kinase (IRAK) to the receptor complex[66]. IRAK is then autophosphorylated and in turn recruits TNF receptor-associated factor 6 (TRAF6). TRAF6 then activates kinases including NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1)[67]. Inhibitor of NF-κB degradation (IκB) is subsequently phosphorylated and degraded, resulting in nuclear translocation of NF-κB[66]. NF-κB belongs to Rel family of DNA binding transcription factor that bind characteristic sequence motifs and activate genes involved in immune and inflammatory response[66]. This is illustrated in Figure 2.

Activation of NFκB stimulates expression of numerous molecules relevant to the pathogenesis of IBD including factors involved in the inflammatory response (IL-1b, TNF, IL-6, IL-8, ICAM1, and other chemokines and adhesion molecules), co-stimulatory molecules (CD40, CD80, CD86, and the inducible T–cell co-stimulator ICOS)[68]. In addition, inhibition of NF-κB can attenuate experimental colitis, illustrating its proinflammatory effect[69]. Other studies have shown that NFκB maintains gut epithelial homeostasis by exerting mucosa protective effects. For example, failure to activate epithelial cell NF-κB *in vivo* results in a significant increase in radiation-induced epithelial cell apoptosis[70]. Indirectly blocking NFκB activation *via* targeted deletion in *MyD88* gene worsens DSS induced colitis in mice in gut epithelial cells, this will be discussed in further detail below[71].

NEGATIVE REGULATORS OF INNATE IMMUNE RESPONSE

TLR signaling in the gut is kept under control by inhibitors of TLR signaling. One such negative modulator is Toll-inhibitory protein (Tollip). Prolonged exposure to LPS (most commensal organisms contain LPS in their cell wall) leads to elevated Tollip expression which makes intestinal epithelial cells poorly responsive to TLR-dependent response to commensal microflora[72]. Other negative regulators of TLR signaling include SIGIRR (single immunoglobulin-IR-related molecule or Tir8) and peroxisome proliferator-activated receptor-γ (PPAR-γ)[73,74]. Consistent with the biological plausibility that reduced expression of these inhibitors lead to IBD, intestinal inflammation is enhanced in SIGIRR-deficient mice[73]. In addition, expression of PPAR-γ is decreased in patients with active UC (but not CD) and up-regulated by 5-aminosalicylic acid[75,76].

Negative regulation of the host innate immune responses to the indigenous microflora maintains gut homeostasis. Key players in the negative mucosal regulation include interleukin-10 (IL-10) and IL-2, as evidenced by mice with deficiencies in these factors develop spontaneous intestinal inflammation, but are protected from intestinal disease when raised in germ-free environments[77-79]. This indicates that at steady state, pathologic consequences of immune activation by commensal microflora are constitutively inhibited by IL-10 and -2 dependent mechanisms. Colitis can be prevented when *IL-10*$^{-/-}$ mice are crossed to MyoD88 deficient mice, demonstrating that IL-10 maintains intestinal immune homeostasis by negatively regulating MyD88-dependent, commensal-induced inflammation[71]. In contrast, colitis in IL-2 deficient mice is independent of MyD88 and TRIF pathway. This indicates that the commensal dependent colitis in IL-2 deficient mice is driven by either nonclassical TLR-dependent signaling (independent of MyD88 and TRIF) or through a non-TLR innate pathway.

The development of colitis in IL-10 and IL-2 deficient mice is mediated by a pathologic T-helper type 1 (Th1) immune response through instructive signals induced upon innate recognition of microbes[79,80]. The Th1 response in IL-10 deficient mice is dependent on MyD88-dependent IL-12 or -23 p40 signaling[71,81]. In contrast, the aberrant Th1 response in the absence of IL-2 does not go through a classical microbial induction of MYD88/TRIF pathway and is also independent of IL-12 or -23 p40 signaling[71]. IL-27, a new bioactive member of the IL-12 cytokine family composed of an IL-12 p40 related polypeptide (EBI3) and a unique p28 subunit, may be the candidate instructive cytokine to drive a MyD88 independent Th1 response[71,82]. Its expression is associated with commensal-dependent, Th1 polarized colitis in IL-2 deficient mice[71]. Interestingly, IL-27 expression also is correlated with *IL-10*$^{-/-}$ Th1 polarized colitis, suggesting that IL-27 is regulated by both the TLR/MyD88 dependent and independent pathway[71].

The differential expression of TLRs may also in part explain how host intestinal mucosa discriminates between commensal (most of which are gram-negative bacteria that contain LPS in their cell wall) and pathogenic bacteria. Oral tolerance is believed to be controlled by antigen-presenting cells in the Peyer's patches by stimulating the activity of regulatory T-cells which suppresses adaptive immune response[83]. These Peyer's patch DCs produce IL-10 in response to inflammatory stimulation such as LPS, which is a ligand for TLR4. Unlike other TLRs, TLR5 is expressed mainly on CD11C+LPMC and not on conventional DCs or macrophages[84]. Interestingly, TLR4

expression is low in CD11C+LPMC[84]. Low expression of TLR4 in CD11C+LPMCs may prevent inappropriate immune response to commensal bacteria whereas TLR4 expression in Peyer's patch DCs leads to IL10 production and immune tolerance to commensal bacteria. In contrast, expression of TLR5 on CD11C+LPMC may allow mucosal inflammation following exposure to pathogenic flagellated bacteria.

ADAPTIVE IMMUNITY

It is established that the recognition of commensal-derived antigens by the adaptive immune system or its stimulation by the innate immune system play a key role in the pathogenesis of IBD. In particular, studies in genetically engineered systems and in inducible and adoptive transfer models of chronic intestinal inflammation have shown a key role of effector T lymphocytes for the inflammatory process in the gut. Importantly, both T helper 1 (Th1) and Th2 T cells have been shown to cause chronic gut inflammation, with CD having a predominately Th1 cytokine profile and UC having a Th2 cytokine profile (Table 2).

Differentiation of naïve T-cells into various T-cell subsets is summarized in Figure 3. IL-12, composed of P40 and p35 subunits, induces the formation of IFN-γ producing Th1 cells. In contrast, IL-4 induces STAT6 activation, promoting the expression of GATA-3, which feed forward to induce IL-4 expression and Th2 cell differentiation. IL-23 (composed of p19 and p40) promotes the development of an IL-17 producing CD4+ helper T cell subset through mechanisms that are distinct from the Th1 (STAT1, STAT4, and T-bet) and Th2 (STAT6) pathways[85,86]. Tregs, an immune-modulating subset of CD4+ T-cells, can suppress the differentiation and function of Th1 and Th2 cells. Interestingly, in the presence of IL-6, Treg-derived TGF-b can induce the differentiation of Th17 cells[87]. These T-cell subsets and their effect in IBD are discussed below.

T-CELL RESPONSES IN CROHN'S DISEASE

Immunologically it is proposed that inflammation in CD is the product of an exaggerated Th1 response mounted by genetically susceptible host in reaction to components of commensal bacteria[88]. The Th1 cytokine profile, including IFN-α, IL-12 (composed of p35 and p40), and TNF-α, is elevated in patients with CD[89-91]. Polarization of naïve T-cells towards Th1 type differentiation occurs through the activation of signal transducer and activator of transcription-1 (STAT1) and its stimulation of transcription factor T-bet, a Th1 "master switch" that upregulates and stabilizes the expression of IL-12[92-94]. IL-12 can amplify Th1 response by upregulating IL-18 on T-cells. IL-18 then stimulates NF-κB and AP1, and in synergy with STAT4 (activated by IL-12), transactivates IFN-α expression. The proposed pathway of TH1 polarization is summarized in Figure 3.

TNF-α and its family member are important in the pathogenesis of CD. Increased expression of TNF-α is seen in the intestinal mucosa of patients with Crohn's disease and Antibodies to TNF-α such as Infliximab decrease Th1 response in parallel with clinical and endoscopic healing[90]. The expression of LIGHT, another member of the TNF family, is also up-regulated in intestinal biopsies from CD patients and stimulation of the LIGHT receptor (herpesvirus entry mediator) induced IFN-γ production in lamina propria T cells, while blocking LIGHT inhibited CD2-dependent induction of IFN-γ synthesis, indicating a role for LIGHT in the regulation of IFN-γ and may be a way in which T-T cell interactions propagate intestinal inflammation[95,96].

TL1A is a recently identified member of the TNF superfamily that interacts with the death domain-containing receptor DR3 and found to be a strong co-stimulator of T-cells. Activation of T-cells by IL-12/IL-18 induces expression of DR3[97]. In T-cells, ligation of DR3 by TL1A activates NF-κB and leads to powerful co-stimulation of IFN-γ[98]. Co-activation is most evident for CD4+/CCR9+ T-cells which are enriched in the lamina propria and intraepithelial lymphoid compartment of the small intestine[99,100]. These T-cells are also enriched in the peripheral circulation of patients with CD and Celiac disease. IL-12 and IL-18 can also augment IFN-γ production in CD4+/CCR9+ T-cells[101]. In addition, peripheral CD4+/CCR9+ T-cells have been shown to express surface membrane (sm) TL1A, which appears to co-stimulate IFN-γ production independent of, but in synergy with IL12 and IL 18 (Figure 3)[102]. A recent study has provided a genetic perspective on the role of TL1A in

Table 2 Cytokine profile in IBD

Innate Immune Response			Adaptive Immune Response		
Cytokine	Crohn's disease	Ulcerative colitis	Cytokine	Crohn's disease	Ulcerative colitis
IL-1β	I	I	IL-5	N	I
IL-6	I	I	IL-13	N	I
IL-8	I	I	IL-17	I	N
IL-12	I	N	IL-21	I	N
IL-18	I	I	IFN-γ	I	N
IL-23	I	N	TL1α	I	?
IL-27	I	N			
TNF-α	I	I			
Light	I	I			
TL1α	I	?			

IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon; I: Increase; N: Normal.

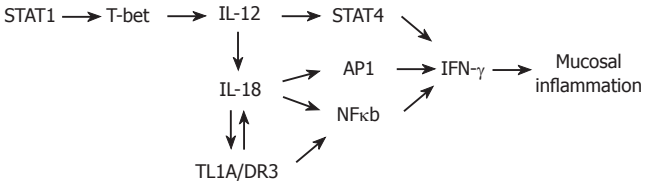

Figure 3 Th1 Polarization Pathway. Polarization of naïve T-cells towards Th1 cell subtype is initially induced by IL-12, a heterodimer of the p40 and p35 subunits. Activation of signal transducer and activator of transcription-1 (STAT1) and its stimulation of transcription factor T-bet, a TH1 "master switch" that upregulates and stabilizes the expression of IL-12. IL-12 can then amplify TH1 response by upregulating IL-18 on T-cells. IL-18 then stimulates NF-κB and AP1, and in synergy with STAT4 (activated by IL-12), transactivates IFN-γ expression.

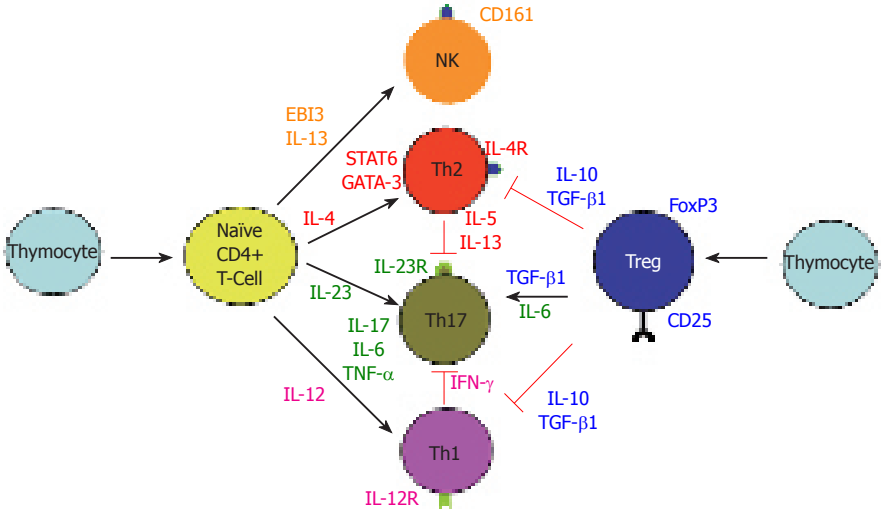

Figure 4 Differentiation of T-cell Subsets. Upon stimulation, naïve CD4+ T-cells differentiate into 3 main subsets, Th1, Th2, and Th17 cell. IL-12 induces the formation of IFN-γ producing Th1 cells. IL-23 promotes the development of an IL-17 producing CD4+ helper T cells. IL-4 induces STAT6 activation, promoting the expression of GATA-3, which feed forward to induce IL-4 expression and Th2 cell differentiation. An EBI3-associated cytokine was hypothesized to be necessary for activation of IL-13 producing NK T-cells. Tregs, an immune-modulating subset of CD4+ T-cells, can suppress the differentiation and function of Th1 and Th2 cells. Interestingly, in the presence of IL-6, Treg-derived TGF-β can induce the differentiation of Th17 cells.

the human TH1 mucosal response in CD. This genome-wide association revealed a highly significant association of SNP haplotypes of the TL1A gene with CD in a large cohort of Japanese patients as well as in two separate, smaller European cohorts[103]. These findings suggest that TNF mediated signaling is an important immune regulatory mechanism in mucosal inflammatory responses.

The IL-23/IL-17 immune axis

A subset of T cells producing IL-6 and IL-17, now known as Th17 cells, has emerged as an important mediator of the T-cell response in gut inflammation (Figure 4). IL-17 is a proinflammatory cytokine that enhances T-cell priming and stimulates fibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple pro-inflammatory mediators such as IL-1, IL-6, TNF, NOS2, metalloproteases, and chemokines[104]. IL-23 (composed of p19 and p40) promotes the development of an IL-17 producing CD4+ helper T cell subset through mechanisms that are distinct from the Th1 (STAT1, STAT4, and T-bet) and Th2 (STAT6) pathways[85,86,105]. Through this pathway, IL-23 regulates inflammatory processes in several mouse disease models[106-108]. In addition, bacterial colonization stimulates IL-23 expression by ileal dendritic cells[109] and the levels of both IL-23 and IL-17 are increased in Crohn's disease tissue[110,111].

CD is thought to be mediated by Th1 cells because of high levels of IL-12 and IFN-γ are detected and treatment with anti-IFN-γ mAb or anti-IL12 (p40) mAB suppresses disease development[112,113]. However, as IL-23 shares the p40 subunit with IL-12, the improved clinical disease may also be an effect of IL-23 inhibition. To determine the contribution of IL-23 in intestinal inflammation, mice with deficiency of IL-23 ($p19^{-/-}$) or IL-12 ($p35^{-/-}$) were used. A recent study showed that the development of colitis was suppressed by IL-23p19 deficiency but not IL-12p35 deficiency in $IL\text{-}10^{-/-}$ mice (model of T-cell mediated gut inflammation)[114]. Administration of IL-23 accelerated the onset of colitis in $Rag^{-/-}$ mice reconstituted with $IL\text{-}10^{-/-}$ CD4+ T-cells[114]. The suppression of inflammation in IL-23p19 deficient mice is not due to impaired Th1 (preserved IFN-γ expression) or Th2 (preserved IL-4 expression) but likely due to IL-17 pathway[114]. Notably, IL-23 (but not IL-12) augments IL-17 and -6 expressions by anti-CD3 mAB-stimulated memory CD4+ T-cells, distinguishing the ability of IL-12 to stimulate naïve CD4+ T-cells[114]. Antibodies that neutralize IL-6 and IL-17 ameliorated the severity of intestinal inflammation in $Rag^{-/-}$ mice reconstituted with $IL\text{-}10^{-/-}$ CD4+ T-cells[114]. These observations indicate that IL-23 promotes development and expansion of a pathogenic IL-6/IL-17 producing memory-activated T-cell population that can trigger the inflammatory cascade leading to intestinal inflammation.

The above findings do not exclude the role of exaggerated Th1 response in CD since IL-12/IFN-γ and IL-23/IL-17 may be parallel pathways involved in inflammatory response. Interestingly, IL-12/IFN-γ and IL-23/IL17 pathways are mutually exclusive, since IFN-γ suppress IL-17 and vice versa (Figure 4)[104]. It is of pathogenic importance to consider all of the major immune pathways that are responsible for the development of CD. For example, therapeutic targeting of only the newly discovered IL-23/IL-17 immune axis may actually exacerbate CD by accelerating the IL-2/IFN-γ Th1 pathway.

T-CELL RESPONSES IN ULCERATIVE COLITIS

In ulcerative colitis, it appears that the T-cell response is Th2 dominant (IL-4, IL-13) and mediated by specialized cells such as NK T-cells. By determining the cytokine profile of lamina propria mononuclear cells (LPMC) isolated from tissue recovered from colonic resection from UC and CD patients, it is found that LPMC from UC patients secreted high amounts of Th2 cytokines IL-13 and IL-5[115]. The IL-13 and -5 LPMC cells bear NK specific markers CD161 and recognizes CD1d, indicating that they are NK T-cells[115]. These NK T-cells are "nonclassical" because they do not express invariant NK T-cell receptors characteristic of most "classical" NK T-cells. They express noninvariant (diverse) TCRs that recognize antigens in association with CD1d, a MHC class I-like molecule present on the surface of dendritic cells

and on non-professional antigen presenting cells (APC) such as intestinal epithelial cells[116]. The "nonclassical" NK T-cells isolated from UC patients exhibited cytotoxicity towards an epithelial cell line (HT-29)[115]. This cell population possibly could be the cells causing epithelial cell cytotoxicity in UC described in 1960-1980s[117]. Together, these data show that ulcerative colitis is associated with an atypical Th-2 response mediated by a distinct subset of NK T-cells that produce IL-13 and are cytotoxic for epithelial cells. However, the extent to which this leads to the ultimate cascade of inflammation in ulcerative colitis remains to be determined.

Colonic epithelial cells express both CD1d and the Epstein-Barr virus-induced gene (EBI3), a protein related to IL-12p40. An EBI3-associated cytokine was hypothesized to be necessary for activation of IL-13 producing NK T-cells[118]. This is consistent with the fact that EBI3 is increased in mucosa from patients with UC as compared with CD or control tissues[119]. In addition, EBI3-deficient mice manifest poor Th2 responses and are resistant to the development of oxazolone colitis, a Th2 colitic murine model[120]. The EBI3-deficient mice have markedly reduced numbers of NK T-cells while the number of both naïve and mature CD4+ and CD8+ cells represerved[120]. These collective data indicate EBI3-associated cytokine (e.g. IL-27) may be necessary for the development of the Th2-cytokine-producing NK T-cells in both UC and oxazolone colitis.

CONCLUSION

Available evidence indicates that IBD is the result of dysregulated immunogenetic parameters that depends on impaired coordination between luminal microorganisms, gut epithelium, and the host immune system in genetically susceptible individual (Figure 1). These immunogenic parameters are complex and biologically divergent, which explains the heterogeneous clinical manifestations and lack of a universal therapeutic response to any single agent. The challenge for the future is to better understand the precise molecular mechanisms of disease immunopathogenesis. This can be achieved by identification of novel immunogenetic parameters, characterization of the heterogeneous parameters, and integrated multiparameter assessment of distinct aspects of the disease biology that are already known. These multi-disciplinary approaches are likely to stratify this complex disease into distinct subgroups that are more biologically homogenous with more defined pathogenic parameters and guidance for a more predictable response for individualized IBD therapy.

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TOPIC HIGHLIGHT

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Role of the intestinal barrier in inflammatory bowel disease

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Abstract

A critical function of the intestinal mucosa is to form a barrier that separates luminal contents from the interstitium. The single layer of intestinal epithelial cells (IECs) serves as a dynamic interface between the host and its environment. Cell polarity and structural properties of the epithelium is complex and is important in the development of epithelial barrier function. Epithelial cells associate with each other *via* a series of intercellular junctions. The apical most intercellular junctional complex referred to as the Apical Junction Complex (AJC) is important in not only cell-cell recognition, but also in the regulation of paracellular movement of fluid and solutes. Defects in the intestinal epithelial barrier function have been observed in a number of intestinal disorders such as inflammatory bowel disease (IBD). It is now becoming evident that an aberrant epithelial barrier function plays a central role in the pathophysiology of IBD. Thus, a better understanding of the intestinal epithelial barrier structure and function in healthy and disease states such as IBD will foster new ideas for the development of therapies for such chronic disorders.

Key words: Intestine; Barrier function; Tight Junction; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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INTRODUCTION

The functioning of the gastrointestinal tract with its balanced microflora depends on the establishment and preservation of distinct compartments which are lined by a sheet of epithelial cells. The gastrointestinal epithelial lining consists of a monolayer of columnar cells that are held together by circumferential intercellular junctions to form a selectively permeable barrier to the luminal contents. Thus, the epithelium prevents unwanted solutes, microorganisms, and luminal antigens from entering the body[1,2]. Components of the intestinal mucosal barrier include luminal secretions such as mucus which is secreted on the apical surfaces of epithelial cells, followed by IECs with lipid plasma membranes, specific membrane transport systems responsible for transepithelial passage of different molecules, and the stromal compartment below the epithelial layer. The intestinal barrier has to be permeable for nutrients and macromolecules which are important for growth and development. At the same time, it has to provide an effective barrier to harmful macromolecules and microorganisms. The mechanisms that have evolved to deal with these physiological events are extremely complex. On the one hand, the structural and functional properties of the epithelium limit the amount of antigens reaching the surface of the epithelium. On the other hand, both specialized cells of the follicle-associated epithelium (M-cells) and dendritic cells sample luminal antigens that are then delivered to the cells of the mucosal immune system thereby guaranteeing permanent immunosurveillance. An intercellular junction referred to as the tight junction (TJ) is located at the apical end of the lateral intercellular space and is considered a key player that regulates paracellular movement of fluid and solutes[3]. Altered TJ structure and epithelial permeability has been observed in IBD[2]. Additionally, pathogens and bacterial toxins influence epithelial permeability by modulating TJ proteins[4-6]. Although the permeability defects could conceivably be due to the marked apoptosis that occurs during the inflammation processes, numerous studies have clearly shown that epithelial cell apoptosis alone does not account for the entire permeability deficits[7-10]. In addition, the importance of the intestinal microflora and more specifically its composition in physiological and pathophysiological processes in the human GI tract is becoming more evident. New discoveries relate to the beneficial effects of normal microflora and probiotics in preventing gastrointestinal infections[11-13]. Probiotics released by bacteria may functionally modulate the intestinal epithelial barrier of the host by different mechanisms, including the competition of whole organisms for contact

with the epithelial surface as well as stabilization of the cytoskeleton and barrier function and the induction of mucin expression.

Some of the most recently available data are discussed in this review. This field is changing rapidly and it is increasingly becoming accepted that immunogenetics play an important role in the predisposition, modulation and perpetuation of IBD. The role of the intestinal environment and the enteric flora in particular appears to be of greater significance than previously thought. This complex interaction of genetic, microbial and environmental factors culminates in a sustained activation of the mucosal immune system. This is facilitated by defects in the intestinal epithelial barrier and its mucosal immune system and results in active inflammation and tissue damage. We will concentrate on the role of the intestinal barrier, its regulation and modulation in IBD.

THE INTESTINAL BARRIER

Mucosal integrity and repair

The small and large intestine have potent mucosal defense and repair mechanisms. These mechanisms include a fast rate of cell renewal, a capable mucosal blood flow, a continuous adherent mucus layer and the presence of regulatory peptides that can stimulate repair mechanisms. The epithelial monolayer which lines the intestinal tract originates from multipotent stem cells present in the crypts[14]. Four major IECs are generated by these multipotent cells: (1) the absorptive enterocytes (reviewed in[15]), (2) the goblet cells responsible for the assembly of mucins[16] and trefoil peptides needed for epithelial growth and repair, (3) the enteroendocrine cells which export peptide hormones (reviewed in[17]) and (4) the paneth cells which secrete antimicrobial cryptidins or defensins, digestive enzymes and growth factors[18]. Injury to the epithelial lining resulting in mucosal erosion/ulceration can occur following exposure to pathogens and chemical therapeutic agents, decreased mucosal defense such as an abnormal mucus layer or reduced production of growth regulatory peptides. The GI tract epithelium has a remarkable capacity to rapidly reseal superficial erosions by migration of epithelial cells, a process referred to as restitution. Epithelial cell proliferation contributes to resealing of larger ulcers. Wound closure is influenced by a diverse array of peptides and growth factors released into the milieu of the regenerating epithelium. For example, the mucosal integrity peptide, TGFα (transforming growth factor α) directly acts on the enterocytes to stimulate proliferation and migration. TGFβ, and the pancreatic secretory trypsin inhibitor (PSTI) protect the overlying mucus layer from excessive digestion by luminal proteases and are involved in maintaining normal mucosal integrity[19]. Epidermal growth factor has been proposed as mucosal protector, playing an important role in luminal surveillance and rapid response to injury[20,21]. Mucosal epithelial cells and paneth cells produce a variety of antimicrobial peptides (defensins, cathelicidins, cryptidin related sequence peptides, chemokine CCL20) and bacteriolytic enzymes (lysozyme, group Ⅱ A phospholipase A2) that protect mucosal surfaces and crypts containing intestinal stem cells against invading microorganisms[21-23]. Trefoil peptides found in the goblet cells of the intestine are a family of three small proteins (TFF1, 2 and 3) which bind to the membrane-anchored glycoproteins of the filamentous brush border glycocalyx of the IECs. These peptides promote cell migration and interact with mucins such as MUC2, suggesting cooperation between the two in epithelial cell protection[24] and preservation of mucosal integrity[25]. Such peptides are rapidly up-regulated at sites of injury and inflammation. Recent experimental data has shown that the physiological role of TFF2, a member of the gastrointestinal trefoil factor family is associated with modulation of the immune system[25] and that the TFF2 rhythm is impaired in cohorts of individuals known to suffer from gastric symptoms, like *H pylori* infection and sleep deprivation[26].

KEY PLAYERS OF THE INTESTINAL BARRIER

Components

Intercellular junctions in epithelial cells play a vital role in regulating mucosal barrier properties. Specifically, the epithelial AJC consisting of the TJ and adherens junction (AJ) (Figure 1) is important in regulating cell-cell adhesion and paracellular movement of fluids and solutes. TJs are continuous, circumferential belt-like structures that form a permeability barrier at the apical end of the intercellular space. TJs regulate vectorial transport of water and electrolytes across the intestinal epithelium and prevent leakage of macromolecules from the gut lumen[27,28]. Additionally, TJs restrict the diffusion of lipids and proteins between the apical and basolateral plasma membranes (fence function) thereby preserving cellular polarity and, in combination with transcellular vectorial transport processes, generate distinct environments in the opposing compartments across the epithelium. Lastly, TJ proteins play an important role in the overall epithelial differentiation of cells, and their deregulation has been observed in epithelial cancers[29-31]. By freeze-fracture electron microscopy, TJs are viewed as a series of anastomosing intramembranous strands, the complexity of which correlates with barrier properties of the epithelium[32,33]. The TJ is a highly dynamic structure that regulates physiologic processes such as glucose absorption[3] and undergoes rapid regulatory changes in response to inflammation[34]. The AJs reside immediately subadjacent to TJs and play an important role in cell recognition and in mediating intercellular associations[35,36]. Both the TJ and AJ are intimately linked in their regulation and function and have therefore been collectively referred to as the AJC. Lastly, subadjacent to the AJC are spot-like intercellular junctions referred to as desmosomes (DMs). Although DMs create strong intercellular associations important in the integrity of stratified epithelia such as the epidermis, the function of DMs in the intestinal epithelium is poorly understood.

At a structural level, all the above intercellular junctions consist of transmembrane proteins that affiliate with the cytoskeleton *via* cytoplasmic plaque proteins. The association of AJC proteins with an underlying perijunctional filamentous actin (F-actin) ring plays an

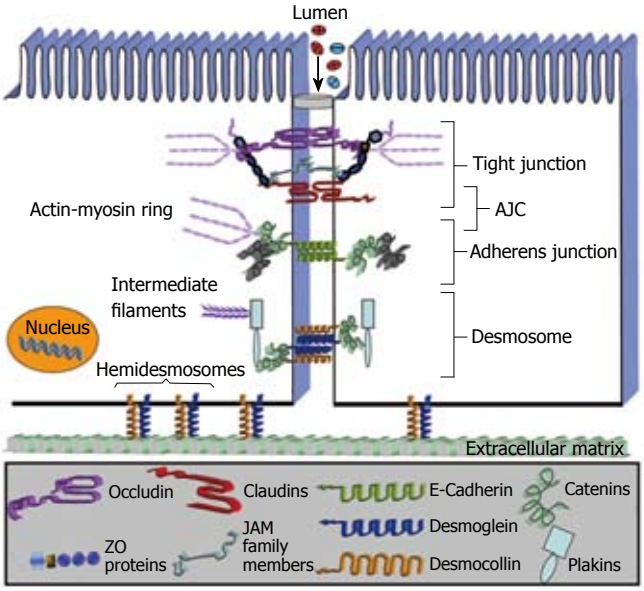

Figure 1 Intestinal epithelial intercellular junctions.

important structural and regulatory role in the AJC[37]. Key integral membrane proteins of the TJ include occludin, claudin family members, junction adhesion molecule-A (JAM-A) and the coxsackie adenovirus receptor (CAR). These proteins are believed to interact in a heterotypic or homotypic fashion in the extracellular space to create the selectively permeable epithelial barrier[37-39]. The intracytoplasmic domains of these TJ proteins interact with scaffolding proteins like the zonula occludens proteins (ZO), which contain several protein-protein interaction domains such as the PDZ domains (PSD 95, Discs large, ZO-1) important in mediating interactions of AJC-associated proteins. E-cadherin is a major transmembrane protein in AJs that associates with the apical perijunctional F-actin ring *via* cytoplasmic proteins in the catenin family[28,36,40]. The intestinal epithelial intercellular junctions are not static extracellular domains. Their adaptive mechanisms can undergo rapid regulatory changes in response to inflammation processes affiliated with IBD. Moreover the appropriate function of the AJC is coordinated by a complex array of signaling proteins which include the Rho and Rap family of GTPases, kinases and phosphatases[9,10,28].

INTESTINAL BARRIER AND REGULATION OF PERMEABILITY IN IBD

Most current treatments for IBD are aimed at reducing disease severity and at prolonging periods of disease-free remission by pharmaceutical suppression of inflammation. It appears that the pathophysiology of IBD is multifactorial. The enormous complexity of IBD pathophysiology therefore requires a systematic approach to identify the molecular events that cause and sustain the chronic, recurring inflammation. It has been suggested that the continuous stimulation of the mucosal immune system due to an increased permeability of the IECs may be the primary defect in patients suffering from IBD, whereas a healthy epithelium provides an effective barrier against luminal antigens. Increased paracellular permeability has been documented in the epithelial lining from both the acutely inflamed and chronically damaged areas of the intestine[41,42]. Animal studies support the tendency for development of inflammation in areas of the intestine lying beneath the permeability defect[43] or even for an increased permeability prior to the onset of a spontaneous intestinal inflammation[44]. It is well known that intestinal permeability is regulated directly through alteration of TJ proteins, or indirectly through effects on the cytoskeleton. A broad array of inflammatory cytokines have been reported to regulate TJs and barrier function by recruiting additional inflammatory cells into the intestinal wall and by re-distribution of TJ proteins in IECs and endothelial cells[45].

CROHN'S DISEASE

Besides genetic linkage like CARD15/NOD2 mutations on chromosome 16[46,47], OCTN1 and 2 mutations on chromosome 5 and DLG5 mutations on chromosome 10[48], abundant evidence indicates that increased intestinal permeability is implicated in the pathogenesis of CD[49]. The integrity of the intestinal barrier in patients with Crohn's disease is known to be compromised. Moreover, first degree relatives of patients with CD have been documented to have increased enteric permeability[50-52]. Interestingly, spouses of patients with CD suffered from increased intestinal permeability[53], which suggests that environmental factors may play a role. It has not been investigated whether these individuals develop CD or other gastrointestinal disorders. However, the debate continues if the increased enteric permeability is to blame for the development of CD or if the barrier defect is a consequence of an existing immune or inflammatory response. Animal models mimicking CD such as the SAMP1/Yit model, showed increased paracellular permeability across intestinal epithelial cells at an early stage of disease (three week old mice), prior to the onset of inflammation[54]. Transgenic animal models revealed the importance of E-cadherin in maintaining the epithelial barrier by demonstrating that dysfunction of AJ proteins contributed to an IBD-like process[55]. In addition, a number of studies have shown a potential role for inflammatory cytokines like TNF-κ and IFN-γ in directly increasing intestinal epithelial permeability. *In vitro* model systems have demonstrated that such pro-inflammatory cytokines influence barrier function by inducing disassembly of TJs in epithelial cells[7,9,10,56]. Furthermore, a role for TNF-κ in both apoptosis independent disruption of epithelial barrier function *via* alteration of TJs and upregulation of epithelial apoptosis (reversible by anti-TNF-κ antibody treatment) in the absence of changes in the expression of TJ proteins was reported, suggesting that TNF-κ may be one of the major links between the leaky bowel and Crohn's disease[57,58]. Soderholm *et al*[59] reported that non-inflamed ileac mucosa from patients with CD showed increased epithelial permeability and that increased endosomal uptake of antigens in ileac CD may be mediated by TNF-κ[60]. A recently published study

pertaining to the expression of TJ proteins (occludin and zonula occludens), alpha2-smooth muscle actin, TGF-κ with a cytoskeletal protein (F-actin) in the intestinal epithelium of patients with inflammatory bowel disease showed that latent dislocation of TJ proteins, without disturbance of the cytoskeleton in the inactive mucosa of patients with CD, may permit the invasion of gut antigens secondary to the functional disruption of TJs, that in turn could initiate an altered immune response[61]. Some reports have demonstrated that IFN-γ increases permeability across model intestinal epithelial cell lines[7,56]. Recently published data suggest that normally poorly invasive enteric bacteria may, in situations of inflammatory stress, exploit lipid raft-mediated transcytotic pathways to cross the intestinal epithelium because of INF-γ-induced disruption of TJs[62]. Although the mechanisms by which IFN-γ induces permeability changes across the epithelium are still incompletely understood, our data demonstrated that the permeability changes occur secondary to endocytosis of TJ transmembrane proteins occludin, JAM-A and claudin-1[9]. Furthermore, we believe that such endocytosis of TJ proteins is initiated by activation of the Rho GTPases and subsequent downstream activation of Rho kinase and actin-myosin II contraction[10].

ULCERATIVE COLITIS

UC is characterized by diffuse mucosal chronic inflammatory disease in the colon. Analogous chronic mucosal inflammation has been observed in animal models such as the mdr1a-/- mice. In this mdr1a-/- animal model a link between epithelial barrier dysfunction and development of colitis has been proposed[63]. Moreover, UC is associated with a mutation of the Toll-like receptor (TLR)-4 gene in humans, resulting in impaired lipopolysaccharide (LPS) signaling[64]. LPS comprises the major cell wall component of gram-negative bacteria and is mainly recognized *via* TLR-4. Stimulation of TLR-4 with bacterial LPS or other ligands[65] leads to activation of the NF-κB signaling system and subsequent induction of inflammatory responses[66]. Kiechl *et al*[67] showed that carriers of these mutations displayed an increased risk of gram-negative infections stressing that the predisposition of UC is associated with a genetic background. Besides these genetic factors, it has been demonstrated that an activated mucosal immune system leads to impaired epithelial barrier function and tissue destruction in patients suffering from UC[6,68]. In contrast to patients with CD, where the cytokines IFN-γ and TNF-κ play a central role in altering the epithelial barrier function, it still remains unclear which panel of cytokines regulates inflammation and induces epithelial barrier dysfunction in UC. Recently published data has shown that IL-13 produced by CD1-reactive natural killer T cells (NKT) played a central role in a murine model of colitis[69]. The IL-13 producing cells were also found in patients suffering from UC which suggests that this cytokine is one of the key mediators in the intestinal pathology of UC. Interestingly Heller *et al* showed that IL-13 mediates a drop of transepithelial resistance without any induction of necrosis in model IECs by increasing the paracellular permeability stressing the profound effect of IL-13 on epithelial barrier function[34]. Furthermore, it has been shown that IL-13 is produced in large amounts in the lamina propria of patients with UC. This is accompanied by increased expression of the pore-forming tight junction molecule claudin 2, which leads to the development of impaired barrier function for small cations and is thought to be responsible for the diarrhea in UC[70].

PROBIOTICS AND BARRIER FUNCTION IN UC AND ANIMAL MODELS OF COLITIS

Data from different *in vitro* and *in vivo* models support the involvement of luminal bacteria in mucosal inflammation and alteration of the intestinal barrier function especially in UC. In contrast to CD, ulcerative colitis is a disease involving mucosa only. Intestinal inflammation is accompanied by direct adherence of bacteria to this mucosal surface while the protective function of the mucus layer seems to be disrupted[71]. Studies have shown that bacteria like *E. coli*, *lactobacilli*, *bifidobacteria* and *streptococci* are able to interact with immunocompetent cells, using the mucosal interface and locally modulate the production of proinflammatory cytokines[72]. They may also directly change the structure of the intestinal epithelial barrier. Although the treatment of CD with probiotics has not demonstrated any sufficient results yet[73-75], the treatment of UC has shown encouraging data[76-81]. Generally the mechanisms by which probiotic microbial agents contribute to the protection of the inflamed intestinal epithelium involve two main categories: (1) competition for binding sites and inhibition of pathogen growth as well as epithelial attachment or invasion[82,83] and (2) stimulation of the mucosal immune system including the stimulation of anti-inflammatory cytokine levels and enhancement of the barrier function. Recent work provided evidence that protective effects of probiotic microorganisms in a DSS model of experimental colitis are mediated by DNA, which was recognized by the mucosal TLR9 receptor and this interaction consequently lead to an increased production of β-defensins. Defensins are known to be responsible for the destabilization and disruption of microorganism cell membranes, leading to an increase in permeability and leakage of small molecules[84,85]. To combat invading pathogens, phagocytes need to be recruited to sites of bacterial entry. Leukocyte recruitment occurs along gradients of chemotactic factors, including chemokines and defensin chemoattractants at nanomolar concentrations[86,87]. In addition, it has been shown that the probiotic compound VSL #3 was effective as primary therapy in a colitis model of IL-10 gene-deficient mice. A direct effect on epithelial barrier function was described. The treatment resulted in a normalization of colonic physiologic function and barrier integrity along with a reduction in mucosal levels of proinflammatory cytokines and a significant improvement in histological disease by secretion of soluble factors enhancing the barrier integrity[88]. This stabilization of the intestinal barrier function is an important target in the treatment of intestinal inflammatory disorders. Otte *et al*[89]

reported that the treatment of cultured IECs with VSL #3 lead to an increase of the transepithelial electrical resistance (TEER). In addition, this probiotic mixture was found to diminish Salmonella-induced alterations in the cellular cytoskeleton[90,91], including the distribution of the tight junction protein ZO-1. The stabilization of the cytoskeleton by regulation of tight junctions is important in the preservation of the epithelial architecture and, thereby, in the maintenance of the intestinal barrier, which might be affected by the MAPK pathway as postulated by the authors[89]. Interestingly, the incubation of IECs with these probiotics also induced the expression of several mucins, leading to less adhesion of microorganisms and its compounds like LPS to the epithelial surface. Together these housekeeping mechanisms might be responsible for the homeostasis of the intestinal barrier function during inflammatory disorders like UC.

CONCLUSION AND PERSPECTIVES

The pathophysiology of intestinal inflammation is multifactorial. Whatever the trigger, increased epithelial permeability plays a central role in the inflammatory process. The permeability changes and inflammation are intimately linked and play a central role in perpetuating the chronic mucosal damage observed in IBD. TJs do not merely represent static junctions between epithelial cells but are multifunctional protein complexes involved in numerous vital and diverse functions of epithelial cells. It is becoming evident that the TJs are extremely dynamic structures involved in developmental, physiological, and pathological conditions like CD and UC, where defects in mucosal integrity and repair remain key elements in initiation and perpetuation of the disease. Future studies will be focused on the mechanisms by which the epithelial barrier can be made "less leaky". The mucosal immune system is the central effector of intestinal inflammation and injury, with cytokines playing a central role in modulating inflammation and epithelial barrier function. Cytokines are, therefore, logical targets for IBD therapy. Finally, preliminary clinical trials of probiotic therapies in IBD may offer a valuable tool for prevention and control of IBD. Understanding the function and action of these probiotics will lead to the selection of useful probiotic strains for clinical application. As more work is directed at the function and modulation of the intestinal barrier, further potential therapeutic targets will provide more options to combat CD and UC.

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Recent trends in the surgical management of inflammatory bowel disease

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Abstract

Surgery is required in the vast majority of patients with Crohn's disease (CD) and in approximately one-third of patients with ulcerative colitis (UC). Similar to medical treatments for IBD, significant advances have occurred in surgery. Advances in CD include an emphasis upon conservatism as exemplified by more limited resections, strictureplasties, and laparoscopic resections. The use of probiotics in selected patients has improved the outcome in patients with pouchitis following restorative proctocolectomy for UC. It is anticipated that ongoing discoveries in the molecular basis of IBD will in turn identify those patients who will best respond to surgery.

Key words: Crohn's disease; Ulcerative colitis; Ileal pouch anal anastomosis; Laparoscopic colectomy

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INTRODUCTION

Significant strides have been made toward more effective medical management of inflammatory bowel disease (IBD) over the past several decades. Surgery, however, is still an essential component of the current multi-modality approach to both Crohn's disease (CD) and ulcerative colitis (UC). The current surgical approach to CD, with its emphasis on bowel conservation, was born out of decades of surgical experience. Early published series on the surgical management of ileal CD supported ileocolostomy with exclusion, the so-called "Berg" or "Mount Sinai" operation, as a safe and often therapeutic surgical approach. Concern that the remaining bypassed segment of diseased intestine placed the patient at risk for recurrent symptomatic CD and adenocarcinoma as well as an incidence of stump blow-out influenced a shift towards resectional techniques. Efforts to resect disease with margins free of microscopic disease gave way to a more conservative approach with the realization that radical resection did not result in lower rates of disease recurrence[1].The addition of strictureplasty techniques to the surgical armamentarium has furthered this trend towards surgical conservatism.

In contrast, the history of the surgical management of UC is marked by a shift from non-resectional strategies to the current emphasis on near-total resection of the colon and rectum. Early approaches to the surgical management of UC involved the construction of appendectomies to facilitate colonic irrigation or diverting ileostomies. The application of proctocolectomy with permanent ileostomy in UC addressed the morbidity associated with not resecting the diseased colon and became standard-of-care for most of the 20th century. The refinement of techniques for constructing a neo-rectum using the ileum in the 1980's led to the wide acceptance of total proctocolectomy with ileal pouch-anal anastomosis (IPAA) as a viable and often preferable surgical option.

Today, 70%-80% of patients with CD and 30%-40% of patients with UC ultimately require surgery. The current indications for surgery in both CD and UC are well described and will be reviewed in this article. In addition, we will focus on more recent developments in the surgical and peri-operative management of IBD including the increasingly broad application of laparoscopic techniques, multimodality therapy for anorectal CD, and the use of post-operative probiotics therapy after IPAA.

CROHN'S DISEASE

The surgical approach to CD is dictated, in large part, by the anatomic distribution of the disease. CD of the distal ileum with a varying degree of colonic involvement is present in 40% of patients. Isolated small bowel disease, isolated colonic, and isolated perineal and anorectal disease are present in 30%, 20%-25%, and 5%-10% of patients respectively. Gastroduodenal disease is not uncommon and has been endoscopically documented in 20% of

CD patients, usually with concomitant ileal disease. Symptoms related to gastroduodenal disease, however, are uncommon, and gastroduodenal disease necessitating operative intervention is rarer still[2].

Irrespective of its anatomic distribution, CD can be classified on the basis of histopathologic and pathophysiologic criteria. The three major subtypes of CD are fibrostenotic disease, which presents with varying degrees of intestinal obstruction, fistulizing disease, and aggressive inflammatory disease. The former two often require operative intervention, while the latter is primarily managed medically.

Operative intervention for CD is indicated when medical therapies fail to adequately alleviate symptoms, or when patients develop one of several complications of the disease; specifically, these include fistula, abscess, obstruction, and malnutrition. Persistent symptoms which compromise quality of life despite several months of aggressive medical management, or recurrent symptoms following repeated attempts to taper aggressive therapies are well described indications for surgery. Adverse reactions to medical therapies, most often steroids, sometimes prompt a more aggressive surgical approach to the control of CD symptoms as well.

Fistula formation is a common component of CD but is infrequently an isolated indication for surgery. Fistulas may be accompanied by abscesses, inflammatory masses, obstructive symptoms, and in rare cases peritonitis, all of which may prompt surgical intervention. Enterocutaneous fistulas and peri-anal or perineal fistulas may significantly compromise quality of life. Operative intervention to alleviate symptoms is often warranted even if these fistulas do not constitute a significant threat to a patient's physical well being. Enteroenteric fistulas are frequently discovered during surgical exploration for other indications. The presence of these fistulas does not mandate surgical repair unless they result in significant sequelae of malabsorption; namely malnutrition, dehydration or extremely frequent bowel movements. Enterovaginal and enterovesical fistulas may necessitate surgery if they are associated with significant symptomatology, vaginosis or urinary tract infections.

Obstruction is a relatively common indication for surgery. The specific character of obstructive symptoms is largely dependent upon the site of obstruction. Most commonly, terminal ileal narrowing causes chronic crampy abdominal pain associated with oral intake, often resulting in malnutrition. Additional less common indications for surgery in CD include acute hemorrhage, malignancy, and fulminant colitis[3].

SMALL BOWEL DISEASE

As stated previously, indications for operative intervention for CD of the small bowel include obstruction and fistula, particularly when accompanied by abscess or an inflammatory mass. When small bowel resections are undertaken, anastomoses should be constructed between segments of bowel grossly free of active disease. In the setting of multiple strictures, strictureplasty may allow for conservation of bowel length. Conventional strictureplasty involves the longitudinal incision of a stenotic segment of bowel with subsequent transverse closure of the enterotomy to increase lumen diameter. Resection and strictureplasty are often used in conjunction with the former technique applied to longer stenotic segments and the latter technique applied to shorter stenotic segments. Strictureplasty techniques aimed at addressing longer stenotic segments have been described in the recent surgical literature and are being applied with increasing frequency[4].

The operative management of small bowel fistulas is a complex subject; the surgical approach is dictated by the structures involved. Generally, fistulous tracts are transected, diseased intestine is resected, the contents of the tracts are evacuated, and necrotic tissue is debrided. In the setting of enterocutaneous fistulas, the opening at the skin is typically excised and allowed to close secondarily. When fistulas involve intra-abdominal organs free of Crohn's disease, the opening in the non-diseased organ is debrided and closed primarily[5].

COLONIC DISEASE

Complications of segmental CD of the colon may be addressed with segmental resection. Diffuse disease may require proctocolectomy with ileostomy, or total abdominal colectomy with ileorectostomy if the distal rectum is free of disease. In the setting of toxic colitis, total abdominal colectomy with construction of an ileostomy and Hartmann pouch is the preferred approach. In principle, the management of fistulous disease of the colon is similar to that described for fistulous disease of the small bowel. Colon with gross disease should be resected. Fistulous tracts to the colon from a diseased small bowel are typically managed with small bowel resection, excision and primary closure of the colonic opening.

ANORECTAL DISEASE

Typical manifestations of anorectal CD include fissures, fistulas, abscesses, and anal canal stenosis. Perianal disease resulting in fistulas and abscesses is frequently treated in stages. Perianal sepsis from abscess formation is addressed with incision and drainage. Subsequent abscess formation may be prevented by placement of seton drains. Fistulotomy and rectal advancement flaps are additional options in the management of chronic fistulas. Fistulotomy is specifically applicable to low fistulas. Rectal advancement flaps are less frequently successful in the CD population and candidates for these approaches must be carefully selected. The absence of significant rectal mucosal disease is a prerequisite for successful advancement flap coverage. The construction of a diverting ostomy may render active disease quiescent and is a useful option in selected patients with debilitating perianal or perineal disease who are poor candidates for a larger operation. Finally, proctectomy and proctocolectomy are appropriate options for patients with persistent, severe disease[6].

ULCERATIVE COLITIS

In contrast to CD, UC may be cured with surgery. The

specific indications for surgical intervention fall into two broad categories: emergent and elective. Emergent indications include massive hemorrhage, toxic colitis, toxic megacolon, and intestinal perforation. Elective indications include the inability of medical therapy to alleviate symptoms, severe malnutrition, the presence of dysplasia, and cancer.

EMERGENCY INDICATIONS

Toxic colitis, characterized by diffuse abdominal tenderness, tachycardia, fever, and leukocytosis is initially treated with fluid resuscitation, intravenous steroids and antibiotics. Surgery is indicated if clinical parameters do not improve with medical therapy in 48 to 72 h. The operation of choice in this setting is a subtotal colectomy and ileostomy with Hartmann pouch or rectal mucous fistula. Pelvic dissection is avoided to allow for a subsequent safe conversion to a proctocolectomy. Toxic megacolon is a variant of toxic colitis characterized by severe dilation of the colon. Toxic megacolon may be acutely managed with a subtotal colectomy and ileostomy. Construction of a skin-level transverse colostomy for decompression and a loop ileostomy for diversion has been described as an alternative approach in this setting. Colonic perforation is typically managed with a subtotal colectomy and ileostomy. Massive hemorrhage is similarly treated with a subtotal colectomy and ileostomy following proctoscopic confirmation that the majority of the bleeding is proximal to the rectum.

ELECTIVE INDICATIONS

Severe persistent symptoms that compromise quality of life are an indication for surgical resection as are complications of long term steroid dependence. Malnutrition and growth retardation are common indications for resection in the pediatric population. Finally, dysplasia or colorectal cancer detected by colonoscopy mandate surgical resection.

Surgical options for chronic ulcerative colitis include proctocolectomy with ileostomy, proctocolectomy with continent ileostomy, and proctocolectomy with IPAA. Proctocolectomy with ileostomy remains an important surgical option with curative potential in UC and relatively low morbidity when compared to proctocolectomy with IPAA. In healthy individuals who are motivated to maintain fecal continence and are willing to accept the potential associated morbidity, restorative proctocolectomy with IPAA is the procedure of choice. Though the necessity of a temporary diverting ileostomy has been called into question by some investigators, the majority of experienced surgeons routinely divert patients undergoing IPAA for a period of two to three months. The construction of continent ileostomies was more popular prior to the refinement of IPAA techniques. Continent ileostomies are prone to failure from valve slippage and this approach is only applicable in highly selected patients. Conversion to a continent ileostomy may be appropriate in patients with an IPAA who develop septic complications but are uncomfortable with the realities of a conventional ileostomy[7].

Table 1 Advances in the surgical management of IBD

Crohn's disease
Laparoscopic ileocecal resection
Multi-modality approach to anorectal CD
Ulcerative colitis
Laparoscopic restorative proctocolectomy
Probiotics

ADVANCES IN THE SURGICAL MANAGEMENT OF IBD

Crohn's disease

The role of laparoscopy in the surgical management of CD has been the focus of considerable investigation over the past decade. Several non-randomized trials suggested equivalent morbidity and mortality following laparoscopic or open ileocecal resection. Benefits of the laparoscopic approach, such as shorter length of hospital stay and lower rates of post-operative bowel obstruction, were suggested in some series. More definitive data in the form of two published prospective randomized trials have shed further light on these topics. In the study published by Milsom *et al*[8], 60 male patients were randomized to either laparoscopic or conventional ileocolic resection. Serial pulmonary function tests (PFT's) were measured post-operatively and were used as an objective surrogate for recovery. While the laparoscopic group's PFT's normalized more rapidly than did those of the conventional surgery group, the return of GI function and length of hospital stay was not significantly different between the two groups[8]. In the study reported by Maartense *et al*[9], sixty patients were randomized to either laparoscopic-assisted or open surgery. Importantly, post-operative care of the enrolled patients, regardless of which operation they received, was standardized. The primary outcome parameter was post-operative quality of life as measured by responses to two standardized questionnaires during a three month follow-up period. Secondary outcomes included operating time, morbidity, hospital stay, post-operative morphine requirement, and costs. Median operating time was longer (115 min *vs* 90 min, $P < 0.003$), median hospital stay was shorter (5 d *vs* 7 d, $P = 0.008$), and costs were lower in the laparoscopic group when compared to the open surgery group. Quality of life did not differ between the two groups[9]. In summary, there is no demonstrable difference in outcomes after open and laparoscopic ileocecal resection. Evidence is somewhat contradictory with regards to recovery time, but there is some evidence suggesting a more rapid recovery following laparoscopic ileocecal resection (Table 1).

As stated previously, 70%-80% of patients with CD eventually require surgery. Unfortunately, rates of disease recurrence remain high with a median time of ten years between a first and second bowel resection. A number of studies have proposed lower rates of disease recurrence after bowel resection when side-to-side stapled anastomoses are performed as opposed to hand-sewn end-to-end anastomoses. The widespread observation that disease recurrence invariably affects

bowel proximal to the prior anastomosis has led some surgeons to hypothesize that some element of relative obstruction at the anastomosis contributes to the pathogenesis of proximal disease. If this is the case, it follows that techniques allowing for a larger, more widely patent, anastomosis might lower recurrence rates. A number of retrospective, and non-randomized prospective studies suggest longer intervals of time prior to second resections when the stapled technique is employed[10]. A multicenter randomized trial is ongoing to investigate these observations.

Several interesting observations and advances have been reported regarding the operative treatment of anorectal CD. As mentioned previously, the placement of a draining seton is of considerable utility in the management of perianal fistulas. Perianal fistula recurrence following the removal of draining setons, however, is common. A combined medical and surgical approach to anorectal disease has been widely advocated. Specifically, the use of infliximab therapy in combination with examination under anesthesia and seton placement is supported by retrospective data published by Regueiro and Mardini[11], and by Topstad *et al*[12]. Irrespective of pharmacologic and surgical intervention, anorectal fistulizing disease remains a difficult problem as evidenced by the 44% rate of fistula recurrence after combined seton placement and infliximab therapy cited in Regueiro and Mardini's study. Surgical diversion is an often effective, if relatively radical, strategy for severe, medically refractory anorectal disease. Galandiuk *et al* reviewed their extensive experience in treating patients with anorectal CD. They identified the presence of anal canal stenosis and concomitant colonic CD as predictors of the need for permanent fecal diversion[13].

Ulcerative colitis

Laparoscopic surgery for UC has attracted considerable interest in recent years. Several case series and case-control studies established the feasibility of laparoscopic restorative proctocolectomy with ileal pouch anal anastomosis[14]. One randomized study comparing hand-assisted laparoscopic and open surgery for both UC and familial adenomatous polyposis has been published[15]. Maartense *et al* randomized sixty patients and documented their post-operative recovery at three months after surgery as measured by two standardized quality of life questionnaires. Secondary parameters included post-operative morphine requirement, operating time, morbidity, hospital stay and costs. Recovery in the two groups was equivalent. The laparoscopic operation took longer and was more costly[15]. At the current time, there is little evidence to suggest a benefit to laparoscopic restorative proctocolectomy as compared to the open operation with the exception of cosmesis. Interest in this approach, however, both in the medical and patient communities, remains great. With increased experience the role for this operation will, presumably, become more clearly defined.

Morbidity associated with restorative proctolcolectomy with IPAA remains significant. Pouchitis is the most common long-term complication following IPAA. This syndrome, most often characterized by increased stool frequency, urgency, and abdominal discomfort, remains poorly understood. The efficacy of antibiotic therapy in the majority of patients suggests an infectious etiology. Promising data supporting probiotic maintenance therapy for relapsing pouchitis after initial treatment with antibiotics was published by Gionchetti *et al*[16]. Forty patients were randomized to receive a probiotic called VSL#3 containing viable lyophilized bacteria including four strains of Lactobacillus, three species of Bifidobacterium and Thermophilus, or placebo. Over a nine month follow-up period, 15% of patients treated with VSL#3 compared to 100% of patients treated with placebo relapsed[16]. Similarly impressive results for VSL#3 were published by Mimura *et al*[17] VSL#3 has also been studied as prophylaxis against pouchitis during the first year following IPAA in a randomized prospective study. Of 20 patients randomized to VSL#3, 2 (10%) developed pouchitis within 12 months. Comparatively, 8 of 20 patients randomized to placebo (40%) developed pouchitis[18]. These results warrant further study of probiotics in the prevention of, and as maintenance therapy following initial therapy for, pouchitis.

CONCLUSION

Surgery remains an important component of the multi-modality treatment of IBD, required in 70%-80% of patients with CD and 30%-40% of patients with UC. Operative intervention for CD is indicated when symptoms are refractory to medical therapies, or when patients develop complications of the disease. Emergent indications for surgical intervention in UC include massive hemorrhage, toxic colitis, toxic megacolon, and intestinal perforation. Elective indications include refractoriness to medical therapy, severe malnutrition, the presence of dysplasia, and cancer. During the last decade significant efforts have been made to apply laparoscopic techniques to the surgical management of IBD. Additionally, significant advances in medical therapy have been made which promise to impact positively on outcomes following surgical interventions. The role of laparoscopy in the surgical management of CD has been the focus of considerable investigation. No significant difference in outcomes after open and laparoscopic ileocecal resection has been demonstrated but shortened recovery time following laparoscopic resection has been suggested in some studies. Except for improved cosmesis, there is little evidence to suggest a benefit to laparoscopic restorative proctocolectomy as compared to the open operation. Morbidity associated with restorative proctocolectomy with ileal pouch anal anastomosis (IPAA) remains significant. Pouchitis is the most common long-term complication following IPAA. Probiotic maintenance therapy for relapsing pouchitis after initial treatment with antibiotics and as prophylaxis against pouchitis during the first year following IPAA is promising and further investigations are warranted.

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TOPIC HIGHLIGHT

Daniel C Baumgart, MD, PhD, *Series Editor*

Special issues in pediatric inflammatory bowel disease

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Abstract

The incidence of pediatric inflammatory bowel disease (IBD) is rising and recent advances in diagnostics and therapeutics have improved the care provided to these children. There are distinguishing features worth noting between early onset and adult onset IBD. Physical and psychosocial development remains a critical target for the comprehensive management of pediatric IBD. Children are not just little adults and consideration must be given to the stages of development and how these stages impact disease presentation and management. The final stage will be the transition from pediatric care to that of adult oriented care and special consideration must be given to make this a successful process. This review highlights special considerations in the management of the child with IBD.

Key words: Inflammatory bowel disease; Crohn's disease

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INTRODUCTION

Inflammatory bowel disease (IBD) first presents in childhood and adolescence in approximately 20% of all cases. A European study reported that in the entire IBD population (children and adults) the incidence of Crohn's disease (CD) increased significantly (+23%), while the incidence of ulcerative colitis (UC) decreased (-17%)[1]. One of the best North American studies reported the incidence of IBD in Wisconsin children to be 7.05 per 100 000, with the incidence for Crohn's disease being 4.56, more than twice the rate of ulcerative colitis (2.14). An equal IBD incidence occurred among all ethnic groups, and children from sparsely and densely populated counties were equally affected and the majority (89%) of new IBD diagnoses were non-familial[2]. The median age of onset is 12 years and there appears to be a slight male predominance in the younger age group. Given the rise in incidence and the onset of disease coinciding with growth and development it is very important to highlight the considerations that should be taken into account when managing childhood onset IBD. This review with underscore the many unique facets involved in the presentation, diagnosis, treatment and psychosocial well being of the child with IBD.

ETIOPATHOGENESIS

The underlying pathogenesis of IBD in children appears to be similar to that in adult onset such that IBD results from a complex interaction of environmental, genetic, and immune factors. Genetics, however, may play an even greater role in disease onset and susceptibility in patients who present earlier in life. Despite research efforts, a gene specific to pediatric onset disease has not been identified. It does appear however that the NOD2 gene is similarly present in both 30%-35% of adult and pediatric CD patients. NOD2 was first identified in 2001. Although the true pathogenic role of NOD2 in CD remains unknown, it is an important gene involved in innate immunity which lends support to the notion that genetically determined defects in innate, and likely adaptive immunity alter the way our mucosal immune system interacts with our resident bacterial flora[3]. This dysregulated interaction leads to the adaptive immune response that is, in large part, responsible for the chronic inflammatory lesions, characteristic of patients with IBD. NOD2 has been well studied in the Caucasian population, however, the frequency of this gene among patients with CD of other racial/ethnic groups in the United States is not well known. Despite similar clinical characteristics significantly lower frequencies of read NOD2 mutations have been seen in African American and Hispanic children with CD compared with Caucasian children with CD[4]. There has been some evidence to suggest that the ethnic background of a child with CD may be associated with different genetic variants. A study from Israel suggested that G908R (SPN 12) allele-variant of the NOD2/CARD15 gene is closely related to the appearance of CD at a young age in Jewish Ashkenazi patients[5]. Research is ongoing to further examine the influence of

ethnicity on disease susceptibility and disease modification. Initial genotype-phenotype correlation studies in children demonstrated that NOD2 is associated with fibrostenosing CD and more rapid progression to surgery[6]. It appears that genetics is only part of the story when it comes to understanding the influences or risk factors at predicting the natural history of disease in pediatric patients. Initial work suggested that there are specific immune markers present in the serum of children with IBD[7]. These markers were initially used to help differentiate CD from UC and to aid in the distinction between functional GI symptoms and those due to underlying IBD, CD in particular. The concept that sero-reactivity to specific microbial antigens seen in a subgroup of patients with IBD likely represents a surrogate measure of an individual's adaptive immune response has resulted in this being an important area of investigation. Since reactivity to yeast oligomannan (anti-*Saccharomyces cerevisiae* antibody; ASCA) was first described in Crohn's disease (CD), the number of CD-specific antigens for which reactivity can be measured has expanded to include the *Pseudomonas fluorescens* related protein (I2), *Escherichia coli* outer membrane-porin (OmpC), and CBir1 flagellin (CBir1)[8-10].

The evolution of serum immune response from diagnostic markers to markers of disease behavior and perhaps prognostic information has resulted in studies that have shown that the presence and magnitude of immune responses in a given child is associated with more aggressive disease phenotypes and more rapid disease progression to complication and surgery. Larger scale studies are underway to confirm these preliminary findings and to further develop a risk assessment tool to identify children at risk of more aggressive disease and subsequent introduction of early intervention[11,12].

DIAGNOSIS

The diagnostic approach to IBD in children is a two-tiered approach. The first diagnostic question is whether the presenting symptoms are compatible with a diagnosis of IBD. In the face of classic, alarm, symptoms the differentiation between IBD and diseases that mimic the symptoms of IBD can be relatively simple. Diarrhea and abdominal pain are the most common symptoms in both CD and UC with rectal bleeding more commonly seen in UC and weight loss and anorexia more characteristic of CD. Perianal disease, like in adults, remains characteristic of CD. However unlike adults, growth failure is an important presenting sign in pediatric onset IBD, most commonly in CD patients. The standard diagnostic approach is employed including routine labs looking for signs of inflammation (ESR, CRP, platelet count) complete blood count with a focus on the hemoglobin for anemia and other labs such as iron panel and albumin to look at nutritional/absorptive state. Stools studies should be done to rule out infection especially in the presence of a travel history. Endoscopic evaluation and tissue diagnosis remains the gold standard[13]. It is recommended that all children undergo both an upper endoscopy and colonoscopy at the time of initial investigation. The findings on upper endoscopy, although often non-specific, may provide additional information in a patient with indeterminate disease of the colon especially if granulomas are found. A recent study found upper gastrointestinal inflammation in 29 of 54 children (22 CD; 7 UC)[14]. Epigastric and abdominal pain, nausea and vomiting, weight loss and pan-ileocolitis were predictive of upper gastrointestinal involvement. However, 9 children with upper gastrointestinal involvement were asymptomatic at presentation (31%). Overall upper gastrointestinal tract inflammation was most common in the stomach (67%), followed by the esophagus (54%) and duodenum (22%). Thus, absence of specific upper gastrointestinal symptoms do not preclude presence of upper gastrointestinal inflammation. It is important to biopsy macroscopically involved and non-involved tissue so to maximize the yield on the initial invasive evaluation. Small bowel radiography is also part of the initial diagnostic evaluation of pediatric IBD patients especially in CD patients. This is especially so for patients in whom ileal intubation was not successful at the time of the colonoscopy or diagnosis is indeterminate. It has been suggested that a normal small bowel radiography alone should not be used to rule out pediatric inflammatory bowel disease when the symptoms suggest it. Colonoscopy with terminal ileal intubation is feasible and safe; it should be attempted in all children with symptoms consistent with inflammatory bowel disease[15]. Radiographic evaluation of the small bowel has evolved to include MRI enterography and CT enterography. Recent advances in gadolinium-enhanced magnetic resonance imaging (G-MRI) have been developed to enhance the resolution of the intestinal mucosa and facilitate the differentiation of UC from CD[16]. Until the MRI scanners are faster and the radiologists are more comfortable interpreting the scans, MRI remains experimental and should be limited to use in centers that have a radiologists who are trained in this technology. CT enterography on the other hand is evolving into the best method of assessing the small bowel and large bowel. The use of Volumen for oral contrast allows for accurate visualization and distension of the mucosa. The radiation is somewhat greater than a standard CT scan however is less than a small bowel follow through with prolonged fluoroscopy[17]. Experience is growing among pediatric gastroenterologists in this area. Positron emission tomography (PET) using fluorine-18-fluoro-deoxyglucose to identify metabolically active tissues was evaluated as a simple noninvasive alternative to conventional studies in identification and localization of active intestinal inflammation in children with IBD[18]. The authors concluded that PET may not be able to replace conventional studies; however, it may be useful when conventional studies cannot be performed or fail to be completed. The cost associated with this test is an important limiting factor. White cell scans have also been proposed as a noninvasive way of evaluating active inflammation, however there can be many false negatives and advances in technology such as the video capsule endoscopy (VCE) provides a unique way of evaluating the small bowel mucosa with increased sensitivity and specificity[19]. VCE may be helpful in patients with persistent small bowel symptoms despite what has been reported as a normal small bowel x-ray particularly those

children with persistent growth failure. Moreover in cases of indeterminate colitis, children may benefit from more detailed small bowel visualization[20].

In the face of diagnostic uncertainty, such as in children presenting with symptoms compatible but not diagnostic of IBD, the pediatric approach tends to be less invasive then the approach to an adult patient presenting with similar symptoms. In this setting pediatricians tend to use non-invasive testing first to gather information that may increase the probability of disease and hence lead to more evidence to support invasive diagnostic testing. Other than routine laboratory tests as noted above, there are fecal markers, such as calprotectin and lactoferrin, and serological markers, such as ASCA and pANCA, that may aid in the differential diagnosis. Earlier reports demonstrated that fecal calprotectin correlated closely with the best invasive measures of colonic and small bowel inflammation in childhood inflammatory bowel disease and lends itself particularly to the monitoring and assessment of therapeutic interventions in children with inflammatory bowel disease[21]. Subsequent experience with calprotectin has yielded similar findings and in some practices is used frequently to differentiate functional from inflammatory GI disorders. Novel technologies have led to the development of a diagnostic algorithm to evaluate the sensitivity and specificity of serological immune markers as predictors of IBD *vs* non IBD. Historically these tests were accurate in differentiating IBD from non-IBD in 2/3 of cases[22]. The addition of novel immune responses and a change in technology may improve the diagnostic accuracy of non-invasive testing to close to 90%. Further validation studies in pediatric patients are needed to determine if indeed these tests will help pediatric gastroenterologist increase the likelihood of diagnosing IBD in the face of positive markers. In the adult cohort that these tests were validated in, it appears that disease was ruled out with approximately 90% certainty. This would be an important advance in pediatrics where invasive testing is avoided if possible.

The next tier in the diagnostic process of IBD is the differentiation between CD and UC. In the face of a classic CD presentation which includes small bowel localization, presence of granulomas, typical endoscopic and histological findings, the differentiation is simple. However there are some features of pediatric onset IBD that may pose more of a diagnostic dilemma when disease is confined to the colon. Despite the classic teachings of UC always involving the rectum, there are reports of rectal sparing in pediatric patients carrying a diagnosis of UC. The group from Boston reported that a significant proportion of children with new-onset UC had patchiness of microscopic features of chronicity (21% of patients), relative (23%), or absolute (3%), rectal sparing, and had little or no crypt architectural distortion in their rectal biopsies (8%). Of interest, these features were not observed in adult patients with UC. In addition, a higher proportion of children with UC initially presented with subtotal or with pancolitis compared with the adults[23]. Another study demonstrated that children < 10 years of age had significantly less crypt branching, plasma cells in the lamina propria, cryptitis, crypt abscesses, and epithelial injury than adults. Endoscopic rectal sparing was also seen in another study in 23% of children with newly diagnosed, untreated UC, and this feature did not correlate with presenting symptoms. However, the presence of rectal sparing may indicate more aggressive disease that is less responsive to medical treatment[24]. These studies must be interpreted with caution as these patients likely have what has become known as UC-like CD and although clinically present with UC symptoms, there are endoscopic and histological features more characteristic of CD. In these cases a more accurate evaluation of the small bowel with video capsule endoscopy may be warranted and perhaps serological immune responses will be helpful in differentiating between CD and UC. Studies have shown that pANCA is more commonly seen in patients with ulcerative colitis and ASCA and OmpC are prevalent in CD patients. It was then found that pANCA may be present in 25% of all CD patients, predominantly in patients with disease confined to the colon. A novel CD marker, anti-CBir1, has been shown to be present in approximately 50% of pANCA positive CD patients but rare (< 5%) of pANCA positive UC patients[25]. This marker may help differentiate CD from UC in indeterminate cases. This may be important as the efficacy of therapies may differ between the 2 pANCA subgroups. Further research is underway to delineate further the role of these markers in predicting response to therapy and post surgical prognosis[26].

TREATMENT

Modern approaches to IBD therapy calls upon the need for disease modifying targets with the goal of mucosal healing . It is hypothesized that mucosal healing should reduce disease related complications and in turn maximize the quality of life of IBD patients. This may be even more of an important issue when treating children with IBD given the long term consequences of early onset aggressive disease presentations. Currently there are few therapies approved for children with IBD, however, the concepts of targeting the full spectrum of the inflammatory cascade and the notion of mucosal healing can be applied. The main goals of targeting therapy in pediatric IBD are to: (1) Maximizing efficacy; (2) Maximize adherence; (3) Minimize toxicity; (4) Maximize quality of life; (5) Maintain physical and psychosocial growth; (6) Prevention of disease complications.

Like their adult counterparts, the current approach to the treatment of children with IBD is based on disease severity. In other words the traditional "step up" approach is applied in most cases. This approach also takes into account medication safety as the more milder/less toxic medications are often employed first letting patients declare themselves failures necessitating navigating up the pyramid to more aggressive anti-inflammatory agents. Pediatric gastroenterologists are limited in their ability to interpret whether this is the correct strategy given few studies have been done in children to support use of these medications especially the Mesalamine based therapies. Often we are forced to extrapolate information from adult study populations which may not be applicable to a child.

Given the potential growth and development implications of persistent inflammation and corticosteroid dependency, efforts are made to maximize both anti-inflammatory and steroid sparing strategies. Aside from the potential growth effects, the esthetic changes associated with corticosteroid use can be devastating to a child. Nutritional therapy is typically unique to pediatric patients however compliance can be an obstacle to administration. A recent prospective, 10-week open-label trial in children with active naive CD randomized children to orally polymeric formula alone or oral corticosteroids. In this small study, children with active and recently diagnosed CD, a short course of polymeric diet was more effective than corticosteroids in inducing healing of gut inflammatory lesions[27]. Further large scaled studies are needed to further evaluate the short and long term benefits of this treatment strategy. In the USA, enteral nutrition is used more so as supplemental nutrition in the face of malnutrition and growth failure.

Recently, there has been a lot of discussion surrounding the notion of turning the therapeutic pyramid upside down, aka "top-down" therapy. In other words in patients who are candidates for corticosteroids, more potent biological therapies, such anti-Tumor Necrosis Factorα (anti-TNFα) therapies, may be considered as alternatives[28]. However the strategy of short term corticosteroids with 6-MP as bridge therapy may be a safer and equally effective strategy in this patient population. Markowitz *et al* demonstrated that a significant proportion of children were off steroids and in remission 600 days after the combination therapy was initiated[29]. If however the desired outcome of a steroid free remission is not achieved in the expected time frame (4-6 months) of this combination then at that time the introduction of a biological therapy should be considered. The hesitation to go directly to a biologic stems from the fact that the thiopurines work well in children and the serious safety concerns. There are definitely safety concerns associated with thiopurines as well, however, TPMT screening and metabolite monitoring enable clinicians to identify at risk patients and dose adjust so to minimize toxicity and dose escalate safely in patients with sub-therapeutic levels and not responding to their current dose[30]. The REACH trial was the first of its kind in pediatrics to evaluate in a multi-center fashion the efficacy and safety of infliximab in 113 patients[31]. The study was not powered for efficacy but the results do support its use in children with the response rate at 10 wk was close to 90% and a remission rate at 54 wk of approximately 50% which includes children off of corticosteroids when receiving drug every 8 wk as opposed to every 12 wk. The safety may have been more favorable among patients receiving the every 12 wk infusions, however the efficacy benefit of every 8 wk may outweigh its safety risks. The data lead to the approval of infliximab for children with luminal Crohn's disease. Clinical trials are underway to evaluate the efficacy and safety of infliximab in children with ulcerative colitis as well as adalimumab for pediatric CD. Weighing the risks and benefits of each therapy must be considered and should be communicated with the child and the family. New safety information has emerged which has already started to alter the approach to patients receiving infliximab. There have been 18 confirmed cases of hepato-splenic T cell lymphoma (HSTCL) reported in patients receiving combination thiopurines and anti-TNFα therapy. Although rare, the majority of cases are fatal which has forced pediatricians to rethink their approach to this patient population. This calls into question of concomitant immunomodulation for the purpose of immunogenicity and perhaps improvement in response rates and how it relates to safety. In REACH all children had to be on concomitant therapy to be eligible so it is not known whether monotherapy would have been as effective. In the adult ACCENT1 trial only one-third of patients were on concomitant thiopurine or methotrexate and at the end of one year the remission rate off corticosteroids was only 24%[32]. Although the median duration of disease at the time of a patient's first infliximab exposure was much lower in REACH as compared to ACCENT 1 and the patient populations are different, one must question as to whether there is an efficacy advantage of the combination as opposed to monotherapy. A significant proportion of children are being removed from thiopurines and continuing the infliximab and some clinicians are extrapolating data from the rheumatoid arthritis literature that suggests that low dose methotrexate (7.5 mg po weekly) is associated with increased through infliximab levels and decreased levels of antibodies to infliximab[33]. There is no data among pediatric patients and further research is needed to validate this strategy. Methotrexate in any form of administration has not been common place in pediatrics. There was always the notion that injections are traumatizing to children and that the oral form may be associated with treatment limiting nausea and the bioavailability was inferior to that when given subcutaneously or intramuscularly. A more recent report however showed that the bioavailability of methotrexate in patients with inflammatory bowel disease is no different from that observed in other disease states[34]. Perhaps the key to deciding the best strategy for an individual patient will be in identifying the at risk patient up front whose risk of untreated progressive disease outweighs the risk of the medications. A recent study reported that imunomodulators are used in approximately 60% of children with CD within 1 year of diagnosis and suggested that lower serum albumin levels and hematocrit, and elevated erythrocyte sedimentation rate at diagnosis may predict the need for immunomodulators earlier in the disease course[34].

Future research will evaluate the role genetics and immune responses as immunological risk factors for aggressive disease. In other words, stratify patients into high risk and low risk which will in turn modify treatment approaches.

SPECIAL CONSIDERATIONS

Bones and growth

Consideration must be given to those issues which are unique to children. Most adult and pediatric gastroenterologists would agree that the presence of growth and pubertal delay is a key factor in the management of pediatric IBD. Puberty is the most dynamic phase of growth in childhood.

Maintaining adequate nutrition, minimizing inflammation and maximizing treatment off of corticosteroids remains an integral part of managing the potential growth stunting effects of active IBD, most specifically small bowel CD. Growth failure has been reported in up to 40% of children with CD and < 10% of UC. On occasion growth and pubertal delay is the only presenting sign of IBD and can precede any GI symptoms[36]. Growth failure is secondary to malnutrition with decreased intake contributing significantly to the malnutrition. Other factors such as increased GI losses, malabsorption, psychosocial factors and medication effects can certainly impact an individual's nutritional state. Ongoing inflammation with release of specific cytokines that suppress growth factors has been shown to be a very important determinate of growth failure. Evidence suggests that IL-6 mediates growth failure in children with Crohn's disease[37]. Nutritional supplementation and need for "catch-up" growth should be an important part of the evaluation of a pediatric IBD patient. A child's self esteem can be significantly impacted by growth failure and every effort should be made to make this a priority in the management plan. Administration of growth hormone was examined in a pilot study (7 patients) and did not demonstrate any effect on growth[38]. However the combination of growth hormone with nutritional supplementation in an IBD patients would likely yield positive results given the important of adequate caloric intake in patients with ongoing inflammation.

Defective bone mass accrual is another complication of chronic inflammatory disease and the major causative factor is growth failure. There are, however, other variables, such as physical activity, altered body composition, and disordered calcium and vitamin D metabolism which certainly play a role in bone mass. Persistent inflammation in the face of IBD can also impact the maintenance of bone mass. The method for assessment of bone mass in children is very important and what is currently used in adults does not apply to the pediatric age group. Bone mineral density (BMD) T-scores are appropriate for individuals that have reached skeletal maturity, but they should not be used in pediatric DEXA reports[39]. Instead, a Z-score should be calculated by subtracting the measured BMD from the expected BMD for individuals of the same age and sex, and dividing the result by the standard deviation. Additional adjustments may be needed in small or physically immature children (e.g., using height age or bone age instead of chronological age for Z-score calculation). It is important to note that a diagnosis of osteoporosis should not be made in children based on DEXA results alone. Experts in the field suggest that a Z-score of < -2.0 in children should be reported descriptively as "reduced bone mineral mass for age"[40]. Because of these limitations, total body bone mineral content may be clinically more useful than BMD in children, especially when studying patients over a longitudinal time frame. Quantitative computed tomography (QCT) is an alternative to measure BMD in both adults and children[41]. QCT offers the advantage of providing a true volumetric BMD, and it can distinguish the individual contributions of cortical and trabecular bone. QCT can be performed in conventional CT scanners, usually in the lumbar vertebrae. It involves a higher radiation dose than

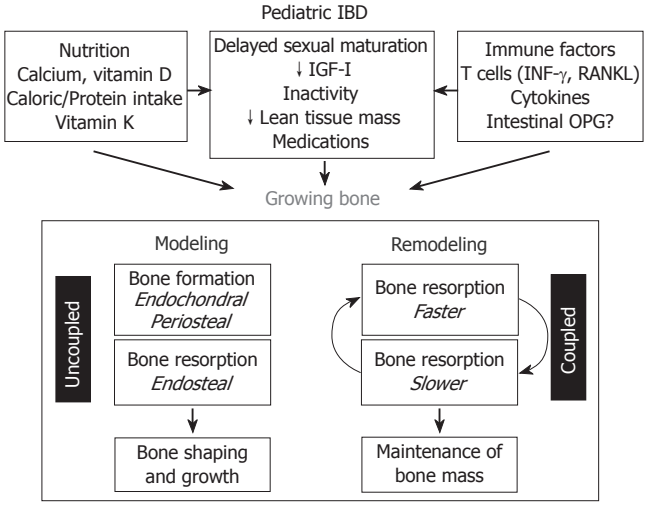

Figure 1 The effect of IBD on skeletal development.

DEXA. CT devices are available that measure BMD in the peripheral skeleton, with minimal radiation exposure, but the clinical significance of these measurements in children and adults with IBD is not known. Other experts in the field have shown that the apparent prevalence of osteopenia in children with IBD differs according to the method of data analysis used. Failure to account for bone age led to a label of moderate or severe osteopenia in 65% of cases. After adjustment for bone age, the proportion of children with osteopenia fell to 22%. Data suggests that children with IBD often have small bones for age because they have growth retardation. When DEXA data are interpreted with adjustment for bone size, most children were found to have adequate bone mass. Correct interpretation of DEXA is important for identifying children who may be at a real risk of osteoporosis[42] (Figure 1).

Quality of life and psychosocial issues

The burden of disease, both physical and psychosocial, imposed on children by Crohn's disease (CD) and ulcerative colitis (UC) can be considerable. In order to asses this burden the IMPACT questionnaire, a disease-specific measure of health related quality of life (HRQOL), was developed and validated for use in children and youth with IBD ages 10 to 18 years inclusive[43,44]. One study reported that the majority of patients perceived an improvement in HRQOL within 1 year of diagnosis and this improvement was in keeping with the overall improvement in disease severity[45]. Hester *et al* asked whether it may prove helpful to ask significant others or caregivers besides the patients themselves when evaluating health and the perception of health of patients[46]. This study reported that parents and children with IBD show high agreement when reporting observable aspects of the child's HRQOL. On the other hand, agreement was lower when it concerns more subjective aspects of HRQOL, such as social functioning and emotions. It is worth noting that children reported fewer problems than their parents. Through the CCFA, summer

camp programs are available for children with CD and UC. The goal of such camp programs is to allow children to participate in normal camp activities surrounded by their peers dealing with similar childhood issues. It has been suggested that camp may provide additional psychosocial benefits such as increased self esteem, altered perceptions and attitudes towards illness[47]. A prospective analysis of quality of life was completed at an overnight camp using the IMPACT Ⅱ questionnaire (Canada and United States) and the State-Trait Anxiety Inventory for Children. These questionnaires were administered to the campers at the beginning and at the end of a 1-wk camp to assess HRQOL and anxiety. No difference was found between anxiety scores before and after camp on either the state or trait anxiety inventories. HRQOL, however, improved after the week of summer camp suggesting that camp may normalize the chronic illness experience[48] (Figure 2).

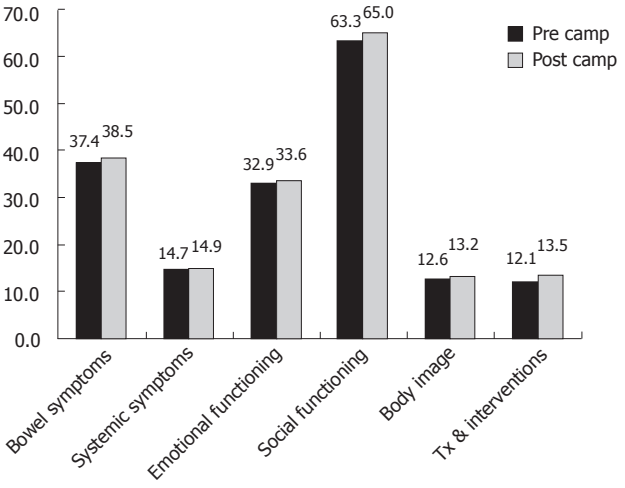

Figure 2 The impact of summer camp on HRQOL in children with IBD.

Psychosocial functioning and medication adherence

When comparing self-reported psychosocial functioning (behavioral/emotional functioning, social competence, self-esteem, stress coping strategies, and social support) of children with IBD to that of healthy children, it appears that most children with mild IBD report normal psychosocial functioning that is similar to that of healthy children[49]. Although rates range, adherence rates among chronic disease in children are typically reported to be approximately 50% with adherence being the lowest in adolescence and when maintenance medications are used even when the disease is in remission[50]. There are multiple factors that impact adherence in pediatric chronic illness which makes it potentially a more complex issue than in the adult population. Parents are often responsible for ensuring their children take their medication, so when evaluating adherence, both the patient and parent must be considered. One study examined the reports of adherence to oral medications, parent-child concordance in reports of adherence, and factors associated with poor adherence in adolescents with IBD. Mean parent- and child-reported adherence scores fell between the "most of the time" and "always" categories, although perfect adherence was low. Among IBD-specific medications < 50% of children and < 40% of parents reported being always adherent to all medications and parent-child concordance was high. Family dysfunction and poor child coping strategies were associated with worse adherence and there appeared to be a trend between more behavioral/emotional problems and lower adherence. Based on these results the authors concluded that adherence should be monitored in families that lack appropriate child discipline and in children who cope by simply wishing stressors would go away[51].

Transition of care

The transition from pediatric to adult medical care of patients with IBD can be difficult for the child and care givers. Clinicians must be sensitive to this transition and the barriers it may present. It has been recommended that the child be approached from the commonly accepted developmental stages, roughly defined chronology by ages 11-13, 14-16, 17-19, and 20-23[52]. These are certain traits needed to make a successful transition of care and allow for the ability to navigate through the adult healthcare milieu. Communication is critical in order for the child and caregiver to anticipate the new roles each member will play in this transition. The process of transition should be a gradual and if the process is delayed the transition may be less successful as the time to prepare and anticipate change has been limited. It is not unusual for the process to be more difficult on the family/primary caregivers than the child themselves. Family members need to relinquish responsibility and the weaning process should begin fairly early in adolescence so that when the time comes to meet with an adult gastroenterologist the patient and caregiver are prepared. In preparation patients may want to put a portfolio together whereby key documents can be brought to their visit to minimize duplication of history taking and perhaps procedures and tests. Examples would include, medical summaries, procedure reports, surgical reports, medication history, recent laboratory results and health insurance information.

Immunization status

Immunosuppressants/immunomodulators have become an integral part of the medical management of IBD both in adults and children. Guidelines have been proposed that take into account the interplay of host susceptibility to infection and the safety and efficacy of immunization for vaccine-preventable diseases. The guidelines suggest that with the exception of live agent vaccines [MMR (measles, mumps, rubella) and Varicella (chicken pox)], most immunizations (killed/attenuated) can be safely administered to patients with IBD even when immune compromised. Moreover, protection against vaccine-preventable illness may be of even greater benefit to those at risk for morbid or lethal complications of infections because of an immune compromised state. It was concluded that for most patients with IBD, recommendations for immunization do not deviate from recommended schedules for the general population[53]. Highlights of the recommendations from this report are the following: (1) Standard recommended immunization schedules for

children and adults should be generally adhered to. (2) At diagnosis, children and adults should have complete review of immunization history for completeness. All patients with incomplete series should commence catch-up vaccination. (3) Children who are not immune by vaccination or acquired immunity through infection should receive varicella vaccine. (4) Live bacterial or viral vaccines should be avoided in immune compromised children with IBD: (a) Treatment with glucocorticoids (prednisone 20 mg/d equivalent, or 2 mg/kg per day if less than 10 kg, for 2 wk or more, and within 3 mo of stopping); (b) Treatment with effective doses of 6-mercaptopurine/azathioprine (effect on safety not established) and within 3 months of stopping; (c) Treatment with methotrexate (effect on safety not established) and within 3 months of stopping; (d) Treatment with infliximab (effect on safety not established) and within 3 months of stopping; (e) Significant protein-calorie malnutrition. (5) Whenever possible, adequate immune response (ie. Tetanus toxoid antibody, H influenza B antibody) should be ascertained for individuals who have required immunization while immune-suppressed. Repeat dosing may be considered when immune response to immunization is insufficient.

CONCLUSION

Approximately one-quarter of IBD presents in childhood, most commonly among the adolescent age group which corresponds to the most dynamic phase of growth. The disease can impact both physical and psychosocial development. Growth failure and bone health are important consideration in the pediatric age group. The diagnosis and treatment requires a comprehensive approach to improve the health related quality of life of children with IBD. Treatments are improving but individualized therapeutic strategies will be a critical part in the management of pediatric IBD so to maximize the therapeutic efficacy and minimize toxicity. Parents and patients must be prepared for the transition from pediatric to adult care and tools are available to improve the success of this process.

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Correlation of integrin β3 mRNA and vascular endothelial growth factor protein expression profiles with the clinicopathological features and prognosis of gastric carcinoma

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Abstract

AIM: To investigate integrin β3 mRNA and vascular endothelial growth factor (VEGF) protein expression in gastric carcinoma, and its correlation with microvascular density, growth-pattern, invasion, metastasis and prognosis.

METHODS: *In situ* hybridization (ISH) of integrin β3 mRNA and immunohistochemistry of VEGF and CD34 protein were performed on samples from 118 patients with gastric cancer.

RESULTS: The positive rate of integrin β3 mRNA in non-tumor gastric mucosa (20%) was significantly lower than that of the gastric cancer tissue (52.5%, χ^2 = 10.20, P < 0.01). In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic or peritoneal metastasis, the positive expression rates of integrin β3 mRNA were significantly higher than those in patients of expanding type (P < 0.01), stage T_1-T_2 (P < 0.01), non-vessel invasion (P < 0.01), without lymphatic metastasis (P < 0.01), without hepatic and peritoneal metastasis (P < 0.01), respectively. In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic or peritoneal metastasis, the positive expression rates of VEGF protein were significantly higher than those in patients of expanding type (P < 0.01), stage T_1-T_2 (P < 0.01), non-vessel invasion (P < 0.01), without lymphatic metastasis (P < 0.01), without hepatic and peritoneal metastasis (P < 0.01), respectively. It was found that the positive expression rate of integrin β3 mRNA was positively related to that of VEGF protein (P < 0.01) and MVD (P < 0.05), meanwhile the positive expression rate of VEGF protein was positively related to MVD (P < 0.05). The mean survival period in patients with positive expression of integrin β3 mRNA and VEGF, and MVD ⩾ 54.9/mm^2 was significantly shorter than that in patients with negative expression of integrin β3 mRNA (P < 0.05) and VEGF (P < 0.01), and MVD < 54.9/mm^2 (P < 0.01). Five-year survival rate in patients with positive expression of integrin β3 mRNA and VEGF, and MVD ⩾ 54.9/mm^2 was significantly lower than those with negative expression of integrin β3 mRNA (P < 0.05), VEGF (P < 0.05), and MVD < 54.9/mm^2 (P < 0.01).

CONCLUSION: Integrin β3 and VEGF expression can synergistically enhance tumor angiogenesis, and may play a crucial role in invasion and metastasis of gastric carcinoma. Therefore, they may be prognostic biomarkers and novel molecular therapeutic targets.

Key words: Stomach neoplasms; Integrin β3; Vascular endothelial growth factor; Metastasis; Prognosis

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INTRODUCTION

Gastric cancer is the leading cause of death in China.

For patients with gastric cancer, metastasis is the most common cause of death and is a major obstacle to successful treatment[1]. The spread of tumor cells from a primary to metastatic site is a complicated and multistage process, which may include cell proliferation and migration, degradation of basement membrane, invasion, and adhesion[2]. Current clinical methods cannot accurately predict which patients will develop metastasis. To develop effective new strategies for the prediction, diagnosis and treatment of metastasis of gastric cancer, molecular mechanisms controlling metastasis must be identified.

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in cancer cell survival, local tumor growth, and development of distant metastasis. The degree of intratumoral microvascular density (MVD) is thought to influence tumor metastasis and consequently prognosis in various human cancers, including gastric cancer[3-6].

Recent investigations have shown that high expression of integrin β3 is positively correlated with invasion and metastasis of cancer cells and tumor angiogenesis[7,8], but only a few studies have investigated the relationship between integrin β3 and prognosis of gastric cancer. In our study, we evaluated integrin β3 mRNA and vascular endothelial growth factor (VEGF) expression in gastric carcinoma and its relationship to pathological markers such as MVD, infiltration, metastasis and prognosis of gastric cancer.

MATERIALS AND METHODS

Patients and specimens

A total of 118 patients (79 male, 39 female; aged 38-80 years, median age 57.8 years) who underwent gastrectomy for gastric carcinoma at Zhejiang Provincial People's Hospital from October 1990 to November 1998 were included in this study. Five-year follow-up data were obtained, and the follow-up ended in November 2003. According to the WHO standard classification (2002), there were 39 tubular adenocarcinomas, 19 papillary adenocarcinomas, 37 poorly differentiated adenocarcinomas 12 mucinous adenocarcinomas, and 11 signet ring cell carcinomas. These patients were also classified into well- and moderately-differentiated (G1 + G2, 70 patients), and poorly-differentiated (G3 + G4, 48 patients) types, based on the predominant differentiation mode, and classified into the expanding (51 patients) and infiltrating (67 patients) type. According to the AJCC TNM staging system (6th ed, 2002), there were 21 stage T_1, 26 stage T_2, 45 stage T_3, and 26 stage T_4; 89 patients with and 29 without vessel invasion; 84 patients with lymphatic metastasis and 34 without; 55 patients with distant metastasis (35 with peritoneal dissemination, 20 with hepatic metastasis) and 63 without. A control study was carried out in 20 samples obtained from non-tumor gastric mucosa 5 cm away from the primary tumor without hyperplasia or atypical hyperplasia.

Reagents

Integrin β3 probe: oligonucleotide probe labeled with digoxigenin was obtained from Chinese Wuhan Boster Biotechnology (MK1602). The target gene mRNA sequence of human integrin β3 is: (1) 5'-GACACCTGTG AGAAGTGCCCCACCTGCCA-3'; (2) 5'-GGATG ACTGTGTCGTCAGATTCCAGTACTA-3'; and (3) 5'-GCTAAATTTGAGGAAAGGCGCGCCAGAGC-3'. Immunohistochemical reagents: first antibody and EnVision kit (DAKO Denmark), mouse monoclonal antibody to VEGF (1:100) and CD34 (1:120); VEGF clone: JH121; CD34 clone: QBEnd 10.

In situ hybridization

The samples in our study were from histological sections from paraffin blocks. *In situ* hybridization was performed according to the manufacturer's instructions. This experiment used RNase-free conditions and APES-treated slides, which were baked at 60℃ for 2-4 h before use. The samples were cut at 4-μm intervals, dewaxed with xylene, hydrated by a series of ethanol solutions (100%, 95%, 80% and 70%); inactivated with endogenous peroxidase using 3% hydrogen peroxide for 10 min at room temperature; washed three times with distilled water, each for 5 min; digested with Pepsin Reagent for 30 min at 37℃; rinsed three times in PBS for 5 min each, rinsed once in distilled water; fixed for 10 min at room temperature using 1% paraformaldehyde plus 1/1000 DEPC, washed three times with distilled water; then incubated in 50 μL pre-hybridization solution for 4 h at 40℃; and then superfluous liquid was absorbed. Each slide was incubated with 20 μL hybridization solution (concentration of the hybridization probe was 2 ng/μL) overnight at 40℃; then washed twice with 2 × SSC at 37℃ for 5 min each, once with 0.5 × SSC at 37℃ for 15 min, once with 0.2 × SSC at 37℃ for 15 min, and once with 0.2 × SSC at room temperature for 15 min. These sections were incubated in blocking solution for 30 min at 37℃ and superfluous liquid was absorbed. Following that, the sections were incubated in mouse biotinylated anti-digoxigenin for 60 min at 37℃, washed three times with PBS for 5 min each; incubated in SABC for 30 min at 37℃; washed three times with PBS for 5 min each; incubated in biotin/peroxidase for 30 min at 37℃; washed three times with PBS for 5 min each; stained with DAB for 3 min at room temperature, and washed thoroughly. The slides were counterstained with Harris hematoxylin, washed, dehydrated in ethanol, cleared in xylene, and enveloped with neutral gum. Negative controls included: (1) hybridization solution without probe; and (2) specimens pre-treated with RNase.

Immunohistochemistry

The EnVision two-step method was done according to the manufacturer's instructions. Tissue sections were cut at 4-μm intervals, dewaxed with xylene, hydrated by a series of ethanol solutions (100, 95, 80 and 70%). Method of high temperature and high pressure (CD34: 0.01 mol/L Sodium Citrate buffering Solution, pH 6.0; VEGF: 0.01 mol/L EDTA buffering solution, pH 9.0) were taken to repair the needed antigen. The sections were washed with distilled water, washed three times with PBS for 5 min each, inactivated endogenous peroxidase using 3% hydrogen peroxide for 10 min at room temperature; washed three times with PBS for 5 min each; incubated overnight with dilute first antibody at 4℃, and rinsed three times with

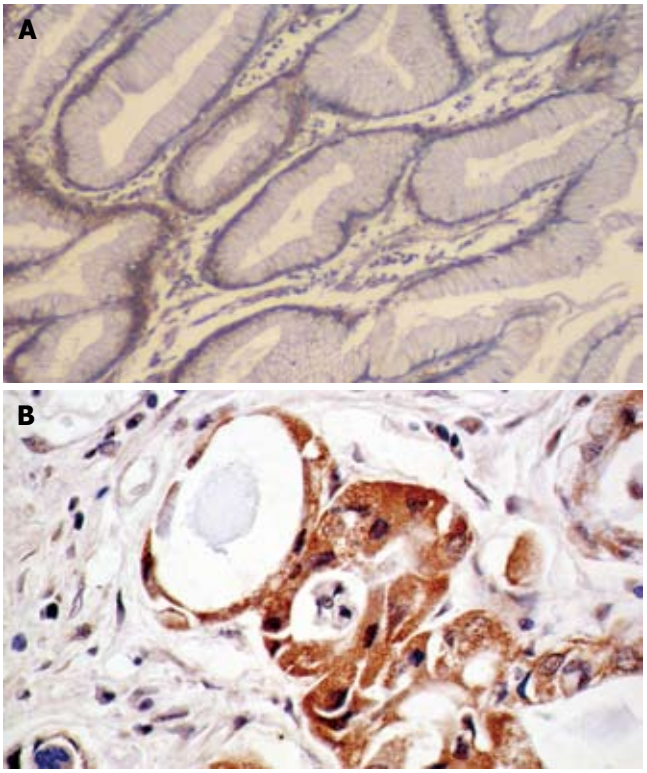

Figure 1 *In situ* hybridization of integrin β3 mRNA. **A**: Negative expression in the plasma of non-tumor gastric mucosa (ISH, × 100); **B**: Positive expression in the plasma of gastric adenocarcinoma (ISH, × 200).

PBS for 5 min each; incubated overnight with goat anti-mouse IgG antibody/HRP polymer for 40 min at 37℃, and washed three times with PBS for 5 min each. Visualization was achieved with DAB for 3 min. After counterstaining with Harris hematoxylin, the slides were dehydrated in 95% and 100% ethanol, cleared in xylene, and enveloped with neutral gum. The negative control included replacement of the first antibody with PBS, and the positive slides provided by the reagent kit were used as the positive control.

Results evaluation

Brown cytoplasm was indicative of positive expression of integrin β3 mRNA. The clearly-stained areas were chosen at high power (× 400), and > 200 cells were quantified in every visual field. According to the percentage of positive tumor cells, all these cells were scored as negative (-) (< 10% or no staining); weak positive (+) (11%-50%); positive (++) (51%-75%); or strongly positive (+++) (> 75%). Positive staining for VEGF was brown in the cytoplasm and/or cell membrane. These cells were scored as negative (-) (no staining); weak positive (+) (< 25%); positive (++) (26%-50%); strongly positive (+++) (> 50%), based on the percentage of positive VEGF cells. Assessment of MVD was performed as follows. Each slide was first evaluated at low power to capture the areas with the highest vascularization. Vessels with a clearly defined lumen or well-defined linear vessel shape, but not single endothelial cells were considered for microvascular assessment. We quantified positive vessels in five chosen fields (× 200) with the highest vascularization[3]. MVD was statistically recorded as the mean ± SD. The patients were divided into a high MVD ($\geqslant$ 54.9/mm^2) and low MVD (< 54.9/mm^2) group based on the mean MVD value (54.9/mm^2) of 118 gastric cancer patients.

Statistical analysis

Statistical analyses were performed using SPSS 10.0 software. Significant differences were compared with Student's *t* test. The χ^2 test was performed on the numerative data. Survival analysis was carried out using the Kaplan-Meier product-limit method, and survival curves were plotted. The differences were evaluated by the log rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Correlation between integrin β3 mRNA expression and clinicopathological features

The positive expression rate of integrin β3 mRNA in non-tumor mucosa was 20% (6/30). Positive signals were distributed in the cytoplasm of non-tumor glandular cell in gastric pit epithelium and the lamina propria, and the majority appeared weakly positive or positive. However, the positive expression rate of integrin β3 mRNA in gastric cancer was 52.5% (62/118). There were significant differences between the two groups ($\chi^2 = 10.20$, $P < 0.01$). Positive cancer cells were stained in the cytoplasm, and most tumor cells infiltrating the muscularis mucosa, serosal layers, extra-serosal layers, and greater omentum positively expressed integrin β3 (Figure 1). In patients of infiltrating type, poorly-differentiated cases, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic and peritoneal metastasis, the positive expression rates of integrin β3 mRNA was significantly higher than those of the expanding type ($\chi^2 = 8.42$, $P < 0.01$), well-, moderately-differentiated case ($\chi^2 = 6.47$, $P < 0.01$), stage T_1-T_2 ($\chi^2 = 26.60$, $P < 0.01$), non-vessel invasion ($\chi^2 = 32.13$, $P < 0.01$), without lymphatic metastasis ($\chi^2 = 31.85$, $P < 0.01$), without hepatic and peritoneal metastasis ($\chi^2 = 20.48$, $P < 0.01$; $\chi^2 = 34.72$, $P < 0.01$) (Table 1).

Correlation between VEGF expression and clinicopathological features

VEGF was rarely expressed in normal gastric mucosa (Figure 2A). Of 118 gastric carcinoma patients, 64 positively expressed VEGF (54.2%), and VEGF staining was mainly located in the cytoplasm of tumor cells and usually observed at the tumor margins (Figure 2B). In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic and peritoneal metastasis, the positive expression rates of VEGF protein were significantly higher than those of the expanding type ($\chi^2 = 10.44$, $P < 0.01$), stage T_1-T_2 ($\chi^2 = 34.19$, $P < 0.01$), non-vessel invasion ($\chi^2 = 39.96$, $P < 0.001$), without lymphatic metastasis ($\chi^2 = 34.71$, $P < 0.01$), without hepatic and peritoneal metastasis ($\chi^2 = 27.25$, $P < 0.01$; $\chi^2 = 52.77$, $P < 0.01$). However, no correlation was found between the different histological types (Table 1).

Correlation between MVD and clinicopathological features

CD34 staining was positive in vascular endothelial cells.

Table 1 Correlation of integrin β3 mRNA, VEGF protein and MDV with clinicopathological features

Clinicopathological parameters	*n*	Integrin β3 mRNA			VEGF				MVD		
		-	+ - + + +	χ^2	*P*	-	+ - + + +	χ^2	*P*	*n*/mm^2	*t*	*P*
Growth pattern	118			8.42	< 0.01			10.44	< 0.01		3.92	< 0.01
Expansive	51	32	19			32	19			49.45 ± 21.72		
Infiltrative	67	24	43			22	45			64.06 ± 18.76		
Histological grade (G)				6.47	< 0.05			3.49	0.062		1.25	> 0.05
G1 + G2	70	40	30			37	33			55.72 ± 21.56		
G3 + G4	48	16	32			17	31			60.70 ± 20.73		
Invasive depth				26.60	< 0.01			34.19	< 0.01		6.41	< 0.01
T_1-T_2	47	36	11			37	10			44.42 ± 19.96		
T_3-T_4	71	20	51			17	54			66.56 ± 17.23		
Vessel invasion				32.13	< 0.01			39.96	< 0.01		7.96	< 0.01
No	29	27	2			28	1			35.68 ± 14.04		
Yes	89	29	60			26	63			64.93 ± 18.07		
Lymph node metastasis				31.85	< 0.01			34.71	< 0.01		8.45	< 0.01
No	34	30	4			30	4			37.60 ± 15.73		
Yes	84	26	58			24	60			66.24 ± 17.24		
Distant metastasis									< 0.01			
No	63	48	15			50	13			42.81 ± 17.23		
Liver metastasis	20	4	16	20.48[a]	< 0.01[a]	3	17	27.25[a]	< 0.01[a]	73.40 ± 8.07	7.65[a]	< 0.01[a]
Peritoneal dissemination	35	4	31	34.72[b]	< 0.01[b]	1	34	52.77[b]	< 0.01[b]	75.79 ± 9.48	10.46[b]	< 0.01[b]

[a]Compared between patients with liver metastasis and those without distant metastasis. [b]Compared between patients with peritoneal dissemination and those without distant metastasis.

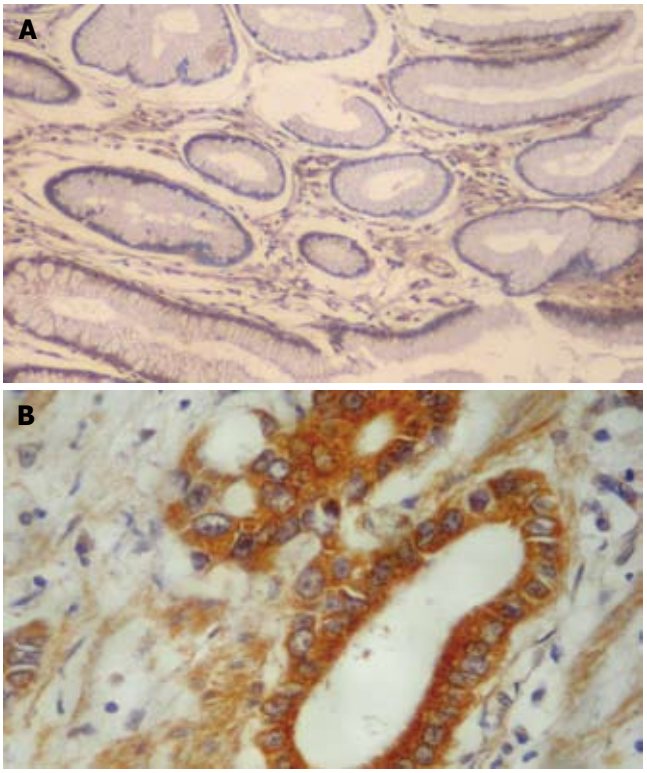

Figure 2 Immunohistochemical staining of VEGF. **A**: Negative staining in the cytoplasm of non-tumor gastric mucosa (EnVision, × 100); **B**: Positive staining in the cytoplasm of gastric adenocarcinoma with greater omentum infiltration (EnVision, × 200).

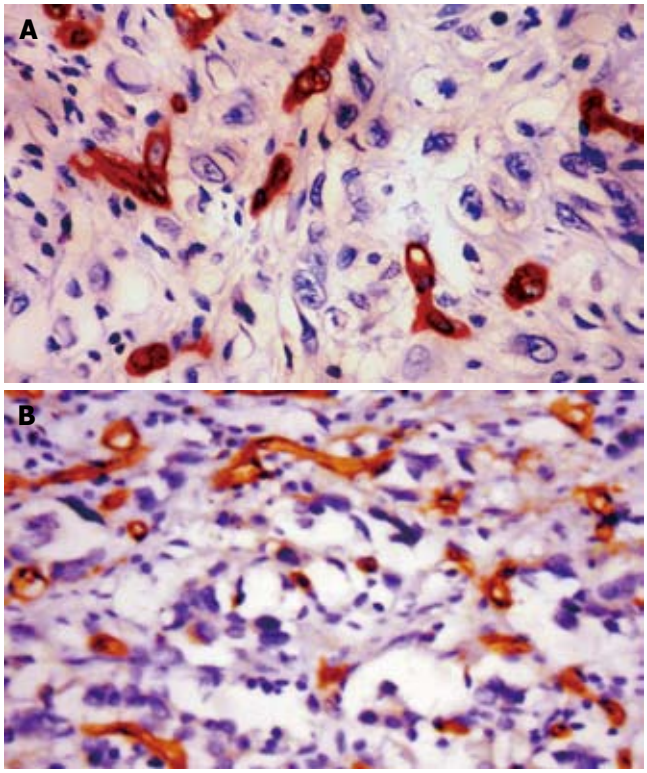

Figure 3 Microvessels in gastric cancer. **A**: Positive staining for CD34 in the vascular endothelial cells of gastric adenocarcinoma with MVD < 54.9/mm^2 (EnVision, × 400); **B**: Positive staining for CD34 in the vascular endothelial cells of gastric adenocarcinoma with MVD ≥ 54.9/mm^2 (EnVision, × 400).

Most microvessels in cancer tissue and surrounding carcinoma were brown or dark brown, strongly stained (Figure 3). In patients of infiltrating type, stage T_3-T_4, vessel invasion, lymphatic metastasis, hepatic and peritoneal metastasis, the mean MVD were significantly higher than those of the expanding type (t = 3.92, P < 0.001), stage T_1-T_2 (t = 6.41, P < 0.01), non-vessel invasion (t = 7.96, P < 0.01), without lymphatic metastasis (t = 8.45, P < 0.001), without hepatic and peritoneal metastasis (t = 7.65, P < 0.01; t = 10.46, P < 0.01), respectively. But no correlation was found within the different histological types (P > 0.05) (Table 1).

Table 2 Correlation of integrin β3 mRNA, VEGF protein and MVD with survival period

Groups		*n*	Mean survival period (mo)	*t*	*P*	5-yr survival rate (%)	χ^2	*P*
Integrin β3 mRNA	-	56	38.46 ± 29.20	2.27	< 0.05	71.4 (40/56)	5.60	< 0.05
	+ - +++	62	27.18 ± 24.73			11.3 (7/62)		
VEGF	-	54	41.70 ± 30.62	3.40	< 0.01	70.4 (16/54)	6.52	< 0.05
	+ - +++	64	24.80 ± 21.78			10.9 (7/64)		
MVD (n/mm^2)	< 54.90	47	44.70 ± 32.05	3.84	< 0.01	63.8 (17/47)	13.85	< 0.01
	⩾ 54.90	71	24.48 ± 20.40			8.5 (6/71)		

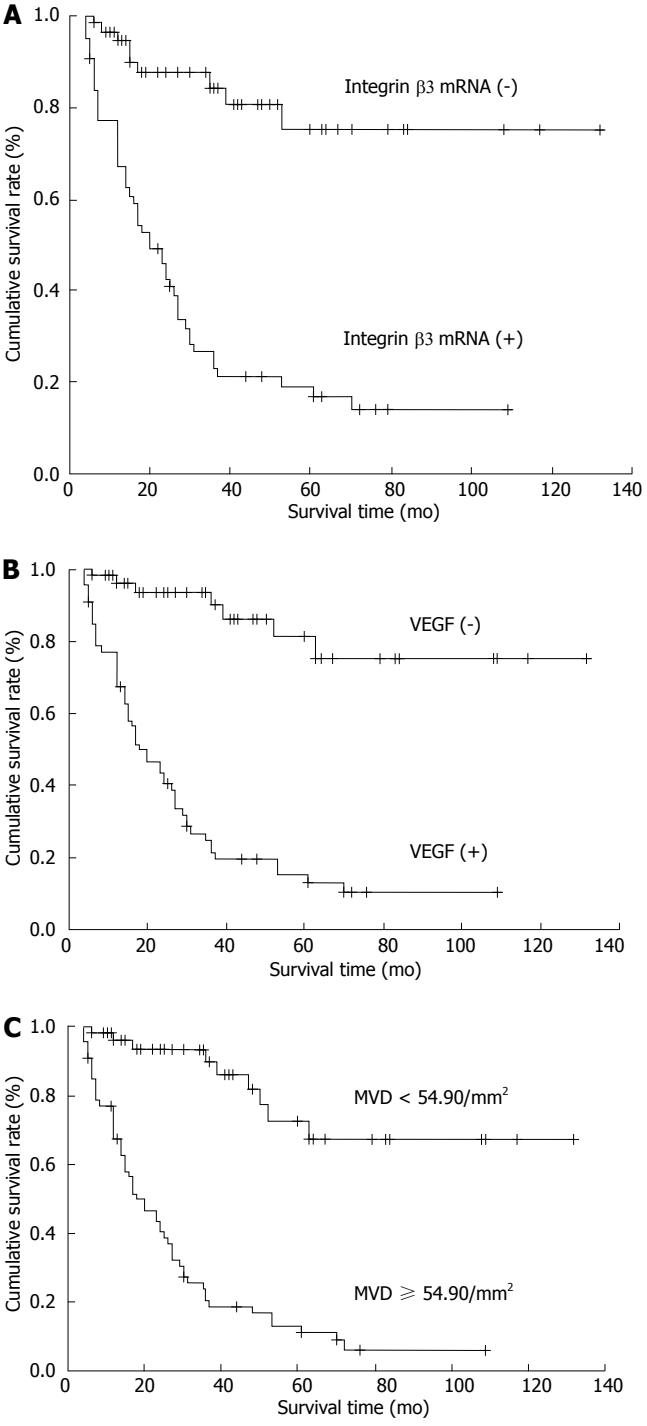

Figure 4 Kaplan-Meier survival curves. **A:** Survival curves with positive and negative integrin β3 mRNA expression in gastric adenocarcinoma ($P < 0.05$); **B**: Survival curves with positive and negative VEGF expression in gastric adenocarcinoma ($P < 0.05$); **C**: Survival curves with MVD ⩾ 54.90/mm^2 and < 54.90/mm^2 in gastric adenocarcinoma ($P < 0.01$).

Correlation between integrin β3 mRNA, VEGF protein expression and MVD

The mean MVD (70.45 ± 14.33/mm^2) in gastric carcinoma specimens with positive expression of integrin β3 mRNA was higher than that of the negative expression group (43.68 ± 18.77/mm^2; $t = 8.78$; $P < 0.01$). Similarly, the mean MVD in gastric carcinoma specimens with positive expression of VEGF (72.96 ± 1.38/mm^2) was higher than that of the negative expression group (37.76 ± 1.84/mm^2; $t = 8.78$; $P < 0.01$). In addition, the rate of VEGF in carcinoma tissues with positive expression of integrin β3 mRNA was 87.1% (54/62), which was significantly higher than that in the integrin β3 mRNA negative expression group (21.4%, 12/56; $\chi^2 = 51.48$; $P < 0.01$). Therefore, there was a positive expression relationship between integrin β3 mRNA, VEGF protein and MVD.

Correlation between integrin β3 mRNA, VEGF, MVD and prognosis

The survival time in patients with positive expression of integrin β3 mRNA, VEGF protein and MVD ⩾ 54.9/mm^2 was significantly shorter than that in patients negative for expression of integrin β3 mRNA ($P < 0.05$), VEGF protein ($P < 0.01$) and MVD < 54.9/mm^2 ($P < 0.01$). The five-year survival rate in patients with positive expression of integrin β3 mRNA, VEGF and MVD ⩾ 54.9/mm^2 was significantly lower than that of patients negative for expression of integrin β3 mRNA ($P < 0.05$), VEGF protein ($P < 0.05$) and MVD < 54.9/mm^2 ($P < 0.01$) (Figure 4A-C; Table 2).

DISCUSSION

Integrins are a major family of cell adhesion molecules, which mediate cell-cell adhesion or cell-extracellular matrix (ECM) adhesion, and affect signal transduction, cell proliferation, differentiation, survival and apoptosis[9]. Integrins are essential for invasion and metastasis of carcinoma cells. Integrins are transmembrane α β heterodimers that require divalent cations for their non-covalent association. To date, 19α and 8β subunits have been identified, and these can combine to form at least 25 different integrins. The ligands of integrins are type I and type Ⅳ collagen, laminin, fibronectin, vitronectin and fibrinogen. Integrins can bind to the ligands in ECM through the specific amino acid sequence in the ligands, which is a tripeptide that features in the integrin-interaction site of many ECM proteins, known as arginine-glycine-aspartic acid (RGD)[10,11]. Different adhesion

receptors play separate roles in intercellular homotypic or heterotypic adhesion, and the connection between cancer cells and ECM[12]. The connection between integrin αvβ3 and fibronectin enables membrane type-matrix metalloproteinase (MT-MMP) to be activated in human endothelial cells, and can also enforce cell invasion[13]. Ria *et al*[14] have found that integrin β3 interacts with fibronectin and vitronectin, which play a role in enhancing proliferation and migration of tumor cells.

Hosotani *et al*[15] have indicated integrin αvβ3 expression is elevated in pancreatic cancer, and expression in patients with lymphatic metastasis is significantly higher than that in patients without lymphatic metastasis. Furthermore, some studies have shown that, in malignant melanoma, ovarian, breast and prostate cancer, the invasive ability of tumor cells with positive integrin β3 expression is powerful, which suggests that integrin β3 is closely related to cancer invasion and metastasis[16-18].

Our study showed that integrin β3 mRNA expression in gastric carcinoma samples was stronger than that in non-tumor gastric mucosa, and the positive expression rate were significantly higher in the infiltrating type group, poor differentiation, perforating serosa layer, lymphoid node invasion, hepatic metastasis and peritoneal dissemination. This result was consistent with previous studies, and showed that increased expression of integrin β3 in gastric cancer influenced the adhesion between tumor cells and ECM. Moreover, it may influence signal transduction, thereby changing the biological behavior of tumor cells, and enhancing the potency of infiltration and migration[19].

Angiogenesis not only accelerates tumor growth, but also increases the opportunity of tumor cells for invading the vasculature, hence it promotes tumor metastasis. As the bridge between vascular endothelial cells and ECM, integrins play an important role in angiogenesis[8,20]. Previous studies have shown that integrin αvβ3 is minimally expressed on resting or normal blood vessels, but is significantly up-regulated in vascular cells within human tumors, and integrin αvβ3 has been implicated in tumor-induced angiogenesis[21,22].

CD34 protein, an endothelial-specific marker, was immunohistochemically stained, and MVD was determined in our study. Statistical analysis showed that β3 integrin mRNA expression ratio was positively correlated with MVD and VEGF. β3 integrin mRNA, VEGF and MVD were all associated with tumor clinicopathological features such as growth pattern, depth of invasion, vessel invasion, and lymph node and distant metastasis, which indicates that VEGF and β3 integrins contribute greatly to the progress of gastric cancer, by modulating angiogenesis. VEGF, the most specific and most potent regulator of angiogenesis, can stimulate the activation of endothelial cells, degradation of the matrix membrane, and migration and proliferation of cells to form new blood vessels[23]. Meanwhile, integrins and ECM are necessarily involved in proliferation and migration of endothelial and vascular smooth muscle cells[24-26]. Integrin β3 may enable VEGF to stimulate endothelial cell proliferation and capillary angiogenesis through activating VEGF receptor-2 (Flk-1) and VEGF receptor-3 (Flt-4)[27,28]. Therefore, integrin β3 and VEGF can enhance synergistically tumor angiogenesis, and lead to proliferation, infiltration and migration of tumor cells.

This study also demonstrated the relationship between integrin β3 mRNA, VEGF protein expression, MVD, survival period, and 5-year survival rate of gastric carcinoma patients. The results suggest positive integrin mRNA and VEGF protein expression, and MVD $\geqslant$ 54.9, are indicative of poor prognosis.

In summary, integrin β3 and VEGF synergistically enhance tumor angiogenesis, and may play crucial roles in invasion and metastasis of gastric carcinoma. Therefore, they can be used as biomarkers for diagnosis and prognosis, and as novel molecular therapeutic targets[29,30].

COMMENTS

Background

Metastasis is the most common cause of death and is a major obstacle to the successful treatment of gastric cancer. It is necessary to develop effective new strategies for the prediction, diagnosis and treatment of gastric cancer metastasis. Angiogenesis plays a central role in tumor growth and development.

Research frontiers

Recent investigations have shown that integrin β3 expression is elevated in pancreatic and breast cancer. High expression of integrin β3 is positively related to cancer cell invasion and metastasis and tumor angiogenesis, but few studies have investigated integrin β3 expression in gastric cancer, and the impact of integrin β3 and VEGF on prognosis.

Innovations and breakthroughs

Integrin β3 and VEGF expression can synergistically enhance tumor angiogenesis, and may play a crucial role in invasion and metastasis of gastric carcinoma.

Applications

This study cannot only define further the mechanism of metastasis of gastric cancer, but can also aid the development of tumor angiogenesis inhibitor. Integrin β3 and VEGF can be used as prognostic biomarkers and as novel molecular therapeutic targets.

Terminology

Angiogenesis is the process that leads to the formation of new blood vessels, and it plays a central role in cancer cell survival, local tumor growth, and development of distant metastasis.

Peer review

The authors studied integrin β3 mRNA and VEGF protein expression in gastric carcinoma by *in situ* hybridization and immunohistochemistry. They concluded that high expression of integrin β3 mRNA and VEGF protein were related to invasion and metastasis, and poor prognosis of gastric cancer.

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COLORECTAL CANCER

Inhibition of signal transducer and activator of transcription 3 expression by RNA interference suppresses invasion through inducing anoikis in human colon cancer cells

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Abstract

AIM: To investigate the roles and mechanism of signal transducer and activator of transcription 3 (STAT3) in invasion of human colon cancer cells by RNA interference.

METHODS: Small interfering RNA (siRNA) targeting Signal transducer and activator of transcription 3 (STAT3) was transfected into HT29 colon cancer cells. STAT3 protein level and DNA-binding activity of STAT3 was evaluated by western blotting and electrophoretic mobility shift assay (EMSA), respectively. We studied the anchorage-independent growth using colony formation in soft agar, and invasion using the boyden chamber model, anoikis using DNA fragmentation assay and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL), respectively. Western blot assay was used to observe the protein expression of Bcl-xL and survivin in colon cancer HT29 cells.

RESULTS: RNA interference (RNAi) mediated by siRNA leads to suppression of STAT3 expression in colon cancer cell lines. Suppression of STAT3 expression by siRNA could inhibit anchorage-independent growth, and invasion ability, and induces anoikis in the colon cancer cell line HT29. It has been shown that knockdown of STAT3 expression by siRNA results in a reduction in expression of Bcl-xL and survivin in HT29 cells.

CONCLUSION: These results suggest that STAT3 siRNA can inhibit the invasion ability of colon cancer cells through inducing anoikis, which antiapoptotic genes survivin and Bcl-xL contribute to regulation of anoikis. These studies indicate STAT3 siRNA could be a useful therapeutic tool for the treatment of colon cancer.

Key words: Colon cancer; Invasion; Signal transducer and activator of transcription 3; Anoikis

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INTRODUCTION

Signal transducers and activator of transcription, STATs, are a family of transcription factors that transmit signals from cell surface receptors directly to the nucleus[1]. Activation of all the STAT proteins is caused by phosphorylation of a single tyrosine residue that leads to dimerization *via* an intermolecular SH2 phosphotyrosine interaction[2-5]. The dimerized STATs then translocate to the nucleus where they regulate gene expression by binding directly to high affinity DNA binding sites or by associating with other transcription factors[6-11]. They play a critical role in mediating cytokine and growth factor signaling involved in cell growth, differentiation and survival[12-14]. Among the seven members of the mammalian STAT family, STAT3 has been the most strongly implicated in oncogenesis[15].

Some studies showed that constitutively activated STAT3 is found in a wide variety of human tumors including multiple myelomas, breast, ovarian, prostate, and head and neck tumors[16-18]. Inhibition of STAT3 signaling with either dominant negative or antisense oligonucleotides against STAT3 suppresses the transformation process in some tumors[19]. Recent studies have shown that treatment of tumor cells with inhibitors of STAT signaling results in decreased cell viability and induction of apoptosis[20]. Together these findings demonstrate that STAT3 signaling

plays a critical role in both the transformation process and tumor progression in some types of cancer.

Recent reports showed that STAT3 has a significant association with tumor invasion and metastasis of a few cancers[21-25]. In renal cell carcinoma, the positive rate of the expression of p-STAT3 correlated well with the depth of tumor invasion and with metastasis[21]. In colorectal adenocarcinoma, p-STAT3 protein was significantly correlated with the depth of tumour invasion, venous invasion, lymph node metastasis, and increasing stages of the Dukes' classification[25]. In addition, blockade of activated STAT3 *via* ectopic expression of dominant-negative STAT3 significantly could suppress angiogenesis, tumor growth, and metastasis in pancreatic cancer[26]. Together these findings demonstrate that STAT3 activation might be a new potential target for therapy of human cancer metastasis. Recently it has been shown that STAT3 is constitutively activated in colon cancer[25], however, the role and mechanism of STAT3 signaling in metastasis of colon cancer remains elusive.

Colorectal cancer is the third most common malignant neoplasm worldwide[27] and the second leading cause of death due to cancer[28]. Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis still remains poor. Liver metastasis is a major cause of morbidity and mortality in patients with colon cancer. Colorectal liver metastasis is associated with a very poor prognosis; most patients die within 2 years of diagnosis despite the availability of numerous therapies. To improve the choice of therapeutic strategy, it is critical that the mechanism of invasion and metastasis of colon cancer be clarified. Here we will show that knockdown of STAT3 expression by siRNA inhibit invasion ability and reduces anoikis resistance in colon cancer cells.

MATERIALS AND METHODS

Human colon cancer cell lines

The human colon cancer cell lines HT29, SW620 and SW480 were obtained from the Institute of Cell Biology, Shanghai, China. All cancer cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). These studies were carried out with Medical Ethical Committee approval of Jiangsu University.

STAT3 siRNA and siRNA transfections

A double stranded siRNA oligonucleotide against STAT3 (5'-AACAUCUGCCUAGAUCGGCUAdTdT-3'; 3'-dTd TGUAGACGGAUCUAGCCGAU-5') was designed and synthesized by Dharmacon Research, Inc. Oligofectamine (Invitrogen, Inc.). In brief, 1 d prior to transfection, cancer cells were seeded, without antibiotics, into a 24 well plate, 1×10^5 cells/well, corresponding to a density of 60%-70% at the time of transfection. There were the following groups: siRNA groups: cells transfected with siRNA at different doses; control groups: cells treated with oligofectamine alone without siRNA. All transfections were performed in triplicate for each time point. At different times after the beginning of the transfection period, all cells were harvested and the following assays were performed.

Western blot analyses

HT29 cells were harvested and lysed in a buffer containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA (pH 8.0), 2 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 mmol/L Na_3VO_4. For western blot analyses, 30 μg of total extracted proteins were applied per lane before SDS-PAGE. Following transfer to nitrocellulose membranes, protein expression levels were detected using polyclonal anti-STAT3 (Santa Cruz, CA), anti- phospho-STAT3, anti-Bcl-xL and anti-survivin (Alpha Diagnostics International, TX). The expression of β-actin (Sigma-Aldrich, MO) was used as a normalization control for protein loading.

Electrophoretic mobility shift assay (EMSA) for STAT3-DNA binding activity

The STAT3-DNA binding activity was assessed by EMSA using the nuclear extract from the three cell lines. The sense strand that binds activated STAT3 protein was 5'-TCGACATTTCCCGTAAATC-3'. Double-stranded oligonucleotide was end-labeled with [γ-^{32P}] ATP using a T4 polynucleotide kinase according to the manufacturer's instruction. The final concentration of probe was 1.75 pmol/L. The labeled probes were then purified by G-25 spin columns. One microliter of ^{32}P-labeled STAT3 oligonucleotide was added to each reaction. For STAT3 specific tests, a 150-fold unlabeled STAT3 probe was applied as a competitor. The final volume of reaction was 20 μL, including 10 μg of nuclear extract and 5 × binding buffer. The reactions were placed on ice for 30 min. The 45 g/L nondenaturing acrylamide gel was pre-run in 1 × TBE buffer at 25 mA for 60 min. After loading of the samples, the gel was run at room temperature in 1 × TBE buffer at 25 mA for 90 min. The gel was dried on a gel dryer, then exposed to X-ray film overnight at -80℃ with intensifying screen. The protein-DNA complex was detected by autoradiography. The Quantity one software was used to analyze the scanned EMSA gel bands.

Anchorage-independent growth assay

For the anchorage-independent growth experiments, HT29 cells (8×10^3 cells/well) were seeded in 0.3% Difco Bactoagar (Difco, MI) supplemented with complete culture medium. This suspension was layered over 0.5 mL of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson, Italy). After 15 d, the colonies were stained with nitroblue tetrazolium, and colonies larger than 50 μm were acquired with a micro-Scopeman camera system (Moritex Europe Ltd, Italy) and analyzed with an Image-Pro Plus (Media Cybernetics, MD) computer program.

Cell invasion assay

Cell invasion assay was performed using Boyden chambers and 8 μm pore size polyvinylpyrrolidone-free polycarbonate filters coated with 25 μg/filter Matrigel (Beckton Dickinson). After transfection for 48 h, Sub-confluent HT29 control or HT29 STAT3 cell lines were harvested by a mild trypsinization, washed twice with Cellect medium and counted. Cells (2×10^5 viable cells/sample) were

allowed to invade Matrigel toward 10% FBS/Cellect at 37℃, 5% CO_2 for 3 h. At the end of the assay, cells on the lower surface of the filter were fixed in ethanol, stained with hematoxylin, and 10 random fields/filter were counted at 200 × magnification. Data represent the average of three experiments, all performed in triplicate.

Induction of anoikis

To prevent cell adhesion, 6-well plates were coated with a solution of polyhydroxyethylmethacrylate (poly-HEMA,Sigma-Aldrich), dissolved at 10 mg/mL in ethanol[29,30]. To coat 6-well plates, 3 mL of poly-HEMA solution was added to each well. Plates were kept at 37℃ for at least 3 d until the solvent had completely evaporated. To induce anoikis, 48 h after transfection, cells were harvested and moved to plates coated with poly-HEMA. 1×10^6 resuspended cells were cultured in DMEM medium containing 15% FCS for 12 h on poly-HEMA-coated dishes at 37℃ and 5% CO_2. Subsequently, cells were gently recovered and submitted to apoptosis detection assays.

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay

The TUNEL assay was performed according to a previous report[31]. Apoptosis index (AI) determination: apoptosis cells/total cell × 100%.

DNA fragmentation assay

For demonstration of internucleosomal DNA fragmentation, after cells were plated in 6-well plates coated with poly-HEMA for different times, the cells were harvested, washed with PBS solution at 4℃, and suspended in lysis buffer (10 mmol/L Tris-HCl pH 7.5, 10 mmol/L EDTA, and 0.2% Triton × 100). After incubation for 15 min at 4℃, samples were centrifuged at 13000 × *g* for 10 min at 4℃. The supernatant containing the fragmented DNA was precipitated with NaCl 0.5 mol/L and 1 volume of isopropanol for at least 1 h at -70℃. Samples were centrifuged at 13000 × *g* for 10 min at 4℃, and the pellet was washed once with 70% ethanol, and air-dried. The precipitates were dissolved in 10 μL TE-RNase (0.1 mg/mL) and incubated at 37℃ for 30 min. Finally, the samples were electrophoresed through a 1% agarose gel.

Statistical analysis

Statistical significance of results was evaluated by SSPS10.0 software. All results with P values $\leqslant 0.05$ were considered significant.

RESULTS

Effects of siRNA on the expression and DNA binding activity of STAT3 in the colon cancer cell line HT29

To inhibit STAT3 expression in colon cancer cells, we used the RNAi method adapted for mammalian cell culture by Elbashir *et al*[32]. A number of 21 bp double stranded RNAs to human STAT3 were synthesized and tested for their ability to knockdown STAT3 expression in colon cancer HT29 cells. The human colon cancer cell line HT29, harboring activated STAT3, was transfected with STAT3 siRNA using oligofectamine. Twenty-four and 72 h after transfection, the protein levels and DNA-binding activities of STAT3 were measured by western blot and EMSA, respectively. Transfection of cells with STAT3 siRNA (Figure 1A) resulted in a highly significant and reproducible decrease in STAT3 expression levels as judged by western blotting (Figure 1B). Figure 1A shows that STAT3 siRNA diminished STAT3 protein compared with control groups, accompanied by a significant decrease in STAT3 DNA-binding activity (Figure 1B). STAT3 knockdown by siRNA was found to be time dependent with the maximum effect achieved at 48-72 h of siRNA treatment (Data not shown).

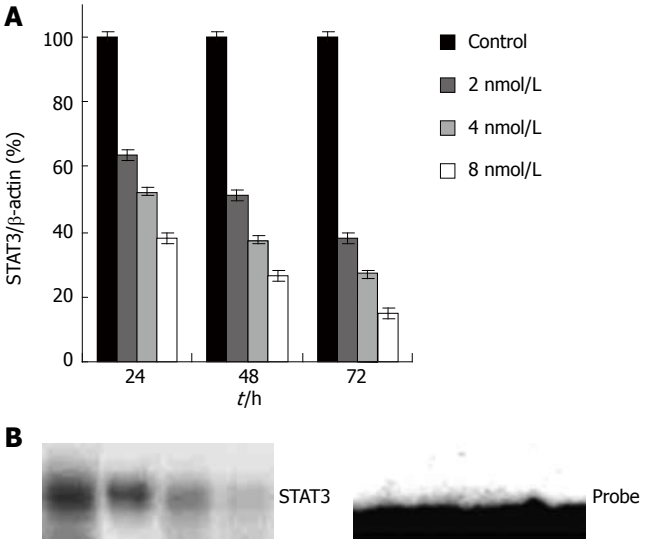

Figure 1 **A**: Expression of STAT3 protein in HT29 cells; **B**: STAT3 DNA-binding activity by EMSA in HT29 cells. 1: Control; 2, 3, 4: siRNA 2, 4, 8 nmol/L.

STAT3 RNAi inhibits anchorage-independent growth in colon cancer cells

Next we evaluated the biological effects of STAT3 suppression in colon cancer HT29 cells by using several different types of assays. Colony formation in soft agar is a property closely associated with malignancy[33]. Figure 2 shows that treatment with STAT3 siRNA induced significant anchorage-independent growth inhibition in a dose-dependent manner.

STAT3 RNAi suppresses invasion in colon cancer cells

Next, we assessed whether STAT3 suppression might decrease the ability of cancer cells to invade the extracellular matrix. HT29 cancer cells were subjected to cell invasion assays, by using Boyden chambers and 8 μm pore size filters coated with Matrigel. Compared with the control group, HT29 cells transfected with siRNA exhibited a scarce ability to cross Matrigel (Figure 3). This difference was statistically significant as compared with control groups ($P < 0.01$).

Effects of STAT3 suppression on anoikis

To explore the molecular mechanisms of invasion of colon cancer, anoikis was studied. To investigate the effect of STAT3 siRNA on anoikis, HT29 cells were subjected to plates seeded in poly-HEMA. After transfection for 48 h,

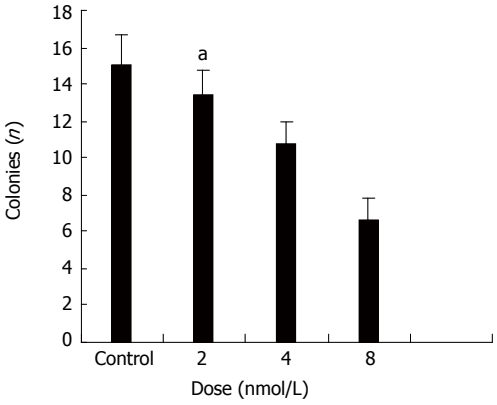

Figure 2 Effects of STAT3 siRNA on anchorage-independent growth of HT29 cells. It shows that treatment with STAT3 siRNA inhibit anchorage-independent growth in a dose-dependent manner. $^{a}P < 0.05$ between siRNA 8 nmol/L and control groups.

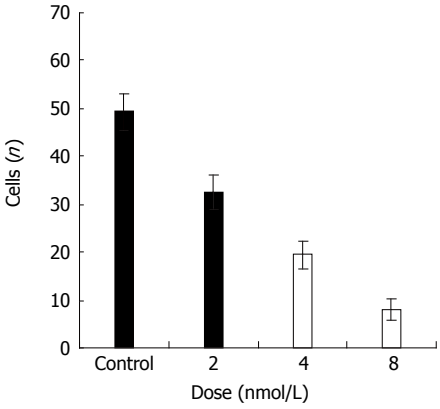

Figure 3 Effects of STAT3 suppression on the invasion ability of HT29 cells.

all cells were removed to plates seeded in poly-HEMA for 12 h. TUNEL and DNA ladder were performed to evaluate the anoikis of HT29 cells. There was little apoptosis in all cancer cells in attached culture. When in a suspended culture, there was significant apoptosis in cancer cells treated with STAT3 siRNA. A significantly higher percentage of apoptotic index was observed in STAT3 siRNA treated cells than in only oligofectamine treated cells (Figure 4A). Consistent with this observation, agarose electrophoresis analysis of HT29 cancer cell extracts showed that there was DNA ladder in cells treated with STAT3 siRNA (Figure 4B). Together these data indicate that siRNA-mediated suppression of the STAT3 gene reduces resistance to anoikis of HT29 cells.

Knockdown of STAT3 expression down regulates survival genes

Recent data indicate that some genes, especially anti-apoptotic genes, contribute to regulation of anoikis[34]. Constitutive activation of STAT3 induces the expression of a number of anti-apoptotic genes including Bcl-xL, a member of the Bcl-2-family of anti-apoptotic genes[20,35], and survivin, a member of the IAP, inhibitors of apoptotic proteins family[36]. Moreover, both of these genes are expressed in colon cancer[37,38]. In order to determine

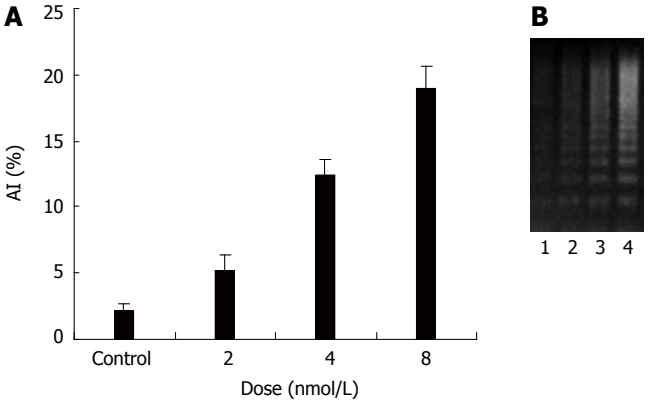

Figure 4 Effects of STAT3 siRNA on anoikis of human colon cancer cell line HT29. **A**: AI (Apoptosis index) induced with STAT3 siRNA in HT29 cells; **B**: DNA ladder by STAT3 siRNA in HT29 cells. 1: Control; 2, 3, 4: siRNA, 2, 4, 8 nmol/L.

whether these two genes might be involved in the STAT3 mediated anoikis resistance in colon cancer, western blot analyses were performed to examine the expression levels of the two genes. Western blot analysis showed that Bcl-xL and survivin protein levels were drastically reduced upon treatment with STAT3 siRNA. Thus, these data show that STAT3 regulates anoikis of at least two distinct antiapoptotic genes in colon cancer HT29 cell (Figure 5).

DISCUSSION

STAT3 is activated by a number of cytokines and growth factors and has diverse functions during embryogenesis and early development[12-14]. Some studies showed that there is persistent activation of STAT3 in a number of human solid tumors including colon cancer[16-18]. Elevated STAT3 activity has been shown to render cells resistant to apoptosis, and inhibition of STAT3 signaling in a number of tumor cell lines with some ways causing a decrease in cell viability and subsequent apoptosis[16,17]. Lots of studies have demonstrated that the transformation process induced by diverse oncogenic protein tyrosine kinases is dependent on STAT3 activation[18,39,40]. Recently it has been shown that STAT3 is constitutively activated in colon cancer. However, the relationships of STAT3 with invasion and metastasis in colon cancer cells remain unclear.

In order to determine the role of STAT3 in colon cancer directly, we have used RNAi to specifically knock down the expression of STAT3, studied the effects of anchorage-independent growth and invasion in the colon cancer cell line HT29. SiRNAs are short oligonucleotides of 21-23 nucleotides in length that can be used *in vitro* to produce sequence specific gene silencing of mammalian cells[32]. It has been shown that siRNAs can be used effectively *in vivo* to suppress gene expression in adult mice[41,42]. SiRNAs can be directly introduced into the CNS to reduce endogenous gene expression[43]. Transfection of colon cancer HT29 cells with STAT3 siRNA resulted in colonies forming membranes with reduced biochemical changes indicative of invasion. In addition, consistent with the results from HT29 cancer cell, SW480 and SW620 cancer cells transfectd with the STAT3 siRNA led to a decrease of both invasion and anchorage-independent

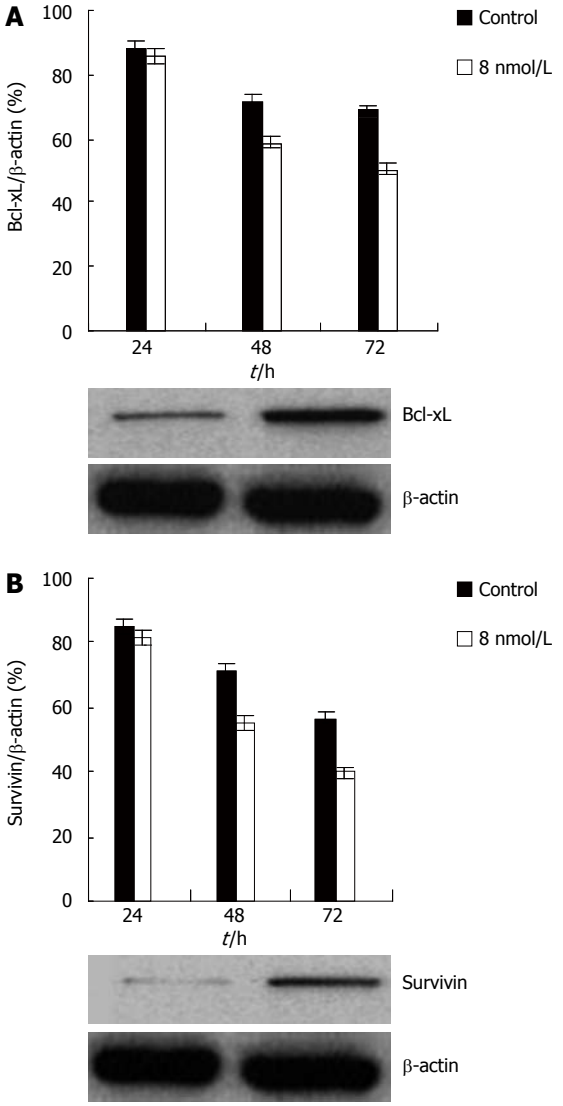

Figure 5 Knockdown of STAT3 expression downregulates Bcl-xL and survivin protein level in the HT29 human colon cancer cell line. **A**: Inhibition of transfection with STAT3 siRNA on Bcl-xL expression of colon cancer HT29 cell; **B**: Suppression of transfection with STAT3 siRNA on survivin expression of colon cancer HT29 cell.

growth. These results are similar to reports previously observed with AG490 inhibiting STAT3 in pancreatic cancer cells[34]. All data suggest that STAT3 down-regulation by RNAi inhibit metastasis of human cancer cell *in vitro*.

The metastasis and invasion of cancer cells were involved in a lot of mechanisms. Anoikis resistance was thought to be high. Anoikis is a peculiar form of apoptosis that is induced by disruption of the interactions between epithelial cells and extracellular matrix[29]. Induction of apoptosis upon loss of anchorage has been termed anoikis (Greek for homelessness)[30]. Anoikis can be considered as a safety program for maintaining normal cell and tissue homeostasis, which prevents survival and reattachment of detached cells to new matrices at inadequate locations. Anoikis has been suggested to act as a physiological barrier to metastasis; resistance to anoikis may allow survival of cancer cells during systemic circulation, thereby facilitating secondary tumour formation in distant organs. Gaining anoikis resistance or anchorage-independent survival is a hallmark of oncogenic transformation. For these experiments, cells are usually seeded in tissue culture dishes that are coated with poly-HEMA, which does not allow cells to attach by preventing matrix deposition. Tumor cells are usually resistant to anoikis[30]. Our results here showed that, in attached culture, neither control cells nor transfected cells exhibited significant apoptosis, suggesting HT29 cells are resistant to anoikis. When seeded in poly-HEMA plates, compared to the control group, there were significant signs of apoptosis in cancer cells treated with STAT3 siRNA. It was shown that clear DNA ladder, the augmented the apoptosis index. These results suggest that STAT3 siRNA induces anoikis of HT29 colon cancer cells.

One mechanism by which STAT3 participates in tumorigenesis is by inhibiting apoptosis through the induction of anti-apoptotic genes such as Bcl-2, Bcl-xL, and Mcl-1[18]. STAT3 responsive elements are found in the promoter region of these genes, suggesting that they are directly regulated by STAT3[16,36]. Interestingly, some reports showed that some anti-apoptotic genes regulate anoikis. Biochemical studies have shown that Bcl-xL, a member of the Bcl-2 family of proteins, induce anoikis of some cancer cells[34,45], and we have found survivin gene is overexpressed in gastric cancer and survivin siRNA reduced the resistance of anoikis of gastric cancer cells (data not shown). In the present study, we have found that both Bcl-xL and survivin are expressed in HT29 cells. Treating HT29 cells with STAT3 siRNA significantly reduces expression levels of both of these genes. These findings suggest that induction of Bcl-xL and/or survivin gene by constitutively activated STAT3 contributes to regulating anoikis of colon cancer cells.

Data presented in this paper are consistent with the growing body of evidence suggesting STAT3 may be an ideal therapeutic target in tumors including colon cancer. We are the first to report that STAT3 siRNA can suppress invasion of colon cancer through inducing anoikis. These results suggest that siRNA may become a useful clinical tool in the future. Since STAT3 signaling is important for the survival of a number of human tumors, STAT3 siRNA could become an effective therapeutic agent for STAT3 dependent tumors.

COMMENTS

Background

Some reports showed that STAT3 has a significant association with tumor invasion and metastasis of a few of cancers, however, the role and mechanism of STAT3 signaling in metastasis of colon cancer remain elusive.

Research frontiers

Induction of apoptosis upon loss of anchorage has been termed anoikis, and anoikis has been suggested to act as a physiological barrier to metastasis. Here we will show that knockdown of STAT3 expression by siRNA inhibit invasion ability by reducing the resistance anoikis in colon cancer cells. In addition, down-regulation of Bcl-xL and/or survivin by STAT3 siRNA might play an important role in inducing anoikis in human colon cancer cell.

Innovations and breakthroughs

The paperwork showed that that STAT3 siRNA can inhibit invasion ability of colon cancer cells through inducing anoikis, and suppression of Bcl-xL and/or survivin by STAT3 siRNA might contribute to regulation of anoikis of human colon cancer cell.

Applications

Here we will show that knockdown of STAT3 expression by siRNA inhibits invasion ability and reduces the resistance anoikis in colon cancer cells. The paper would help to clarify the mechanism of invasion and metastasis of colon cancer and improve the choice of therapeutic strategy.

Terminology

Anoikis (Greek for homelessness) is a peculiar form of apoptosis that is induced by disruption of the interactions between epithelial cells and extracellular matrix.

Peer review

This is an interesting paper investigating the *in vitro* effect and mechanisms of STAT3 siRNAs in a colorectal cancer (CRC) cell line. The main finding of the study was that STAT3 siRNA can inhibit the invasion ability of colon cancer cells through inducing anoikis, in addition antiapoptotic genes survivin and Bcl-xL may contribute to regulation of anoikis.

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BASIC RESEARCH

Effect of Daxx on cholesterol accumulation in hepatic cells

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Abstract

AIM: To study the effect of Daxx on cholesterol accumulation in hepatic cells.

METHODS: Sprague Dawley (SD) rats were fed a normal or high fat diet for 6 wk, and serum lipids and Daxx expression of hepatic tissues were measured by immunoblot assays. $HepG_2$ cells were transfected with the pEGFP-C1/Daxx or pEGFP-C1 plasmid. Cells stably transfected with Daxx were identified by RT-PCR analysis. Total cholesterol levels were determined by high performance liquid chromatography. Activated-SREBP and caveolin-1 were assayed by western blotting.

RESULTS: Hepatic Daxx protein was higher in normal rats than in high fat diet-fed rats. Noticeable negative correlations were seen between Daxx and LDL-C (γ = -7.56, P = 0.018), and between Daxx and TC (γ = -9.07, P = 0.01), respectively. The total cholesterol of $HepG_2$/GFP-Daxx cells was lower than that of control cells or $HepG_2$/GFP cells (9.28 ± 0.19 *vs* 14.36 ± 4.45 or 13.94 ± 2.62, both $P < 0.05$). Furthermore, in $HepG_2$/GFP cells, the expression of activated SREBP was lower than that of control cells, whereas caveolin-1 expression was higher.

CONCLUSION: Overexpression of Daxx in $HepG_2$ cells decreased intracellular cholesterol accumulation, which might be associated with inhibition of SREBP activity and an increase in caveolin-1 expression.

Key words: Daxx; Cholesterol; Hepatic cells; Sterol regulatory element-binding protein; Caveolin-1

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INTRODUCTION

Daxx was first identified as a death-associated protein capable of binding the cytosolic domain of fas, an apoptosis-inducing member of the tumor necrosis factor (TNF) receptor family[1]. Daxx co-localizes with Promyelocytic Leukemia Protein (PML) within nuclear promyelocytic oncogenic domains (PODs)[2,3]. PML and/or POD-associated proteins may function as an important cofactor in governing nuclear hormone receptor transcriptional activity and function[4,5]. Recent studies[6,7] implied Daxx could negatively modulate androgen receptor (AR) transcriptional activity. Androgens affect lipogenic gene expression not only in tumor cells, but also in normal androgen target tissues *in vivo*[8]. AR can directly upregulate sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) by binding an androgen response element in intron 8 of the SCAP gene[9]. Activated SREBP can increase the mRNA and protein levels of genes involved in fatty acid (fatty acid synthase and acetyl-CoA-carboxylase), and cholesterol synthesis (HMG-CoA-reductase and farnesyl diphosphate synthase)[10]. These results indicate that Daxx could possibly regulate cellular cholesterol metabolism by the SREBP pathway.

In the present study, we investigated the correlations between Daxx expression and cholesterol accumulation in liver cells. The findings herein show that overexpression of Daxx in $HepG_2$ cells may decrease intracellular cholesterol, which may be associated with inhibition of SREBP activity related to cholesterol synthesis and an increase in caveolin-1 expression related to excretion.

MATERIALS AND METHODS

Materials

Modified Eagle medium (MEM) and fetal bovine serum

were purchased from Gibco BRL. An antibody (Santa Cruz) directed against active SREBP was used to detect the activation of SREBP, and polyclonal anti-Daxx antibody or anti-caveolin-1 antibody (Santa Cruz) was used to assay the respective protein expression. The plasmids of pEGFP-C1/Daxx and pEGFP-C1 were gifts from Dr. Yanping[11]. The pEGFP-C1/Daxx contains a full-length cDNA of hDaxx in pEGFP-C1 vector. All reagents were of analysis grade.

Animal and diets

Male Sprague Dawley (SD) rats (210 g ± 10 g) were obtained from the animal laboratory of Nanhua University. The animals were individually housed in plastic cages in a temperature (23℃ ± 2℃) and light (alternating 12 h periods of light and dark) controlled room. The rats were randomly divided into two groups. The control group was fed a normal diet, and another group was fed a high-fat diet (15%, lard, wt/wt, HFD) for 6 wk. Rats were allowed free access to food and deionized water throughout the test period. At the end of the experiment, the rats were anesthetized with ketamine and injected with 150 IU of heparin per kilogram of body weight. Fifteen minutes later, the rats were sacrificed by disarticulation. Blood samples were taken from the neck into glass tubes, and serum was obtained by centrifugation (2000 × *g* for 10 min at 4℃). The livers were removed and rinsed with physiological saline. All samples were stored at -70℃ until use.

Estimation of serum lipids

For determination of serum total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), triacylglycerol (TG) concentration, and high density lipoprotein-cholesterol (HDL-C), the corresponding diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used according to the manufacturer's instructions.

Cell culture and transfections

$HepG_2$ cells, a human hepatocyte cell line, were obtained from Zhong Shan University (Guangzhou, China). The cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin, at 37℃ in a humidified incubator containing 5% CO_2. Cells were seeded at a density of 1×10^5 cells/well in a 24-well plate and cultured for 24 h to 60%-80% confluency. To obtain stable transfectants, $HepG_2$ cells were transfected with the pEGFP-C1/Daxx or pEGFP-C1 plasmid using Lipofect 2000 Plus reagent (Invitrogen) in serum-free medium for 4 h at 37℃, according to the manufacturer's recommendations. The transfection medium was removed, and fresh complete growth medium was added. After 24 h post-transfection, the cells in two wells were split into 10-cm dishes in a medium containing 500 μg/mL geneticin (G418; Amresco, Solon, USA), and the medium was changed every 3 d until G418-resistant colonies were clearly evident. Individual colonies were transferred into 6-well plates to continue incubation with G418 selection medium. Individual colonies were evaluated for Daxx expression by Immunofluorescent Microscopy, and a monoclonal cell line was used for all experiments successively.

Reverse transcription-PCR

Total RNA was extracted from the cells using Trizol reagent (Gibco BRL) according to the manufacturer's protocol. Three micrograms of total RNA were used for reverse transcription in a total volume of 20 μL with the SuperScript preamplification system (Promega, Madison, MI). Aliquots of 2 μL cDNA were subsequently amplified in a total volume of 25 μL using the GeneAmp PCR kit (Promega) following conditions recommended by the manufacturer. The sense and antisense primers for Daxx were 5'-TGGCGCTCTATGTGGCAGAGATC-3' and 5'-CTGCATCTGTTCCAGATCCTCCT-3' (829 bp); the sense and antisense primers for actin that were used as an internal control were 5'-GGTGGCACCTGTGGTCCACC T-3' and 5'-CTTCACTTGTGGCCCAGATAG-3' (420 bp), respectively. The cycling conditions were as follows: 94℃ for 5 min, followed by 28 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 1 min, and a final extension of 72℃ for 10 min. PCR products were separated on the 1.5% agarose gel viewed by ethidium bromide staining. These data were acquired with Alpha Imager 2200 software.

Lipid analysis by high performance liquid chromatography (HPLC)

Cells were scraped from culture flasks into 0.9% NaCl (1 mL per 50 cm^2 flask) and homogenized by sonication for 10 s on ice. The protein concentration of cell lysate was determined by a bicinchoninic acid (BCA) kit. An equal volume of freshly prepared cold (-20℃) KOH in ethanol (150 g/L) was added. The cell lysate was repeatedly vortexed until clear. An equal volume of hexane-isopropanol 3:2 (v/v) was then added. The mixture was vortexed for 5 min, followed by centrifugation at 800 × *g* (15℃ for 5 min). The extraction procedure was repeated twice. The combined organic phase was transferred to clean tapered glass tubes and thoroughly dried under nitrogen at 40℃. The tubes were allowed to cool to room temperature. One hundred μL of isopropanol-acetonitrile 20:80 (v/v) was added. The sample was solubilized in an ultrasound water bath at room temperature for 5 min. After centrifugation at 800 × *g* for 5 min, the samples were introduced into the HPLC device using an Agilent 1100 series. Cholesterol was eluted at a flow rate of 1 mL/min, temperature of 40℃ using an eluent consisting of isopropanol-acetonitrile 20:80 (v:v), and detected by UV-absorption at 206 nm[12].

Western blot analysis

Liver tissues excised from rats were analyzed by western blot with an antibody directed against Daxx. $HepG_2$ cells were washed with PBS, and then 0.5 mL of TME lysis buffer (10 mmol/L Tris, pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, and 25 mmol/L NaF) containing fresh 100 μmol/L Na_3VO_4, 20 μg/mL leupeptin, 1 μg/mL pepstatin A, 4 μg/mL aprotinin, and 1 mmol/L DTT were added. Cell lysates were prepared by freezing and thawing of the cells on ice, and subsequent scraping and sonicating for 30 s. The cell lysates were centrifuged for 30 min at 15 000 × *g*. Protein concentrations in the supernatants were determined by a BCA protein assay kit, and the samples were stored at -80℃. For western blot analysis, 20 μg of protein was

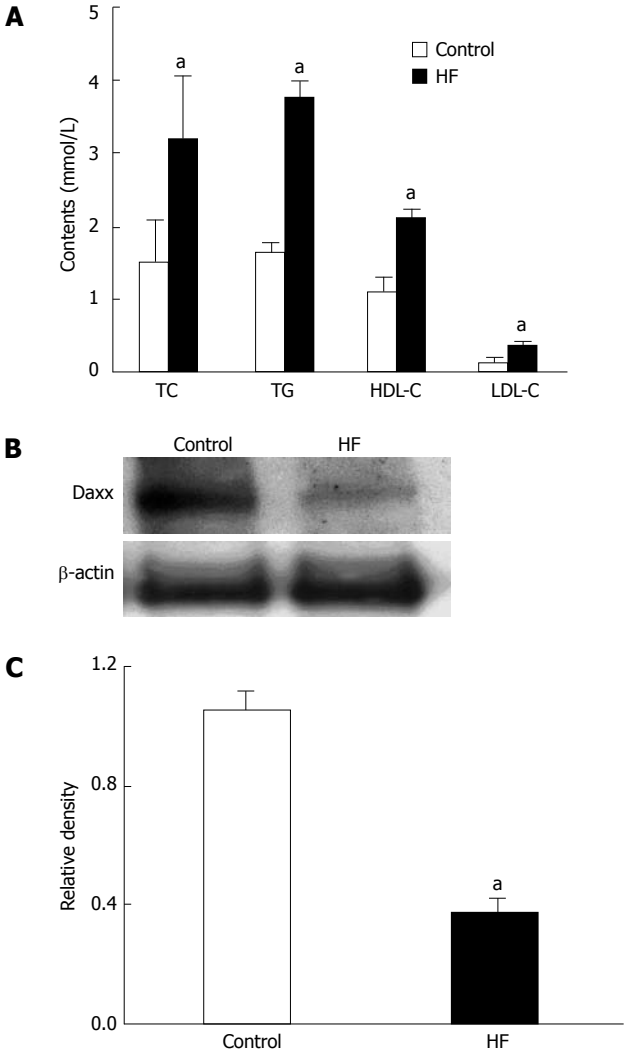

Figure 1 The correlation between Daxx expression of Hepatic tissues and serum cholesterol. **A**: The effect of control or high fat (HF) food on serum cholesterol and triglycerides in rats; **B**: Hepatic Daxx expression of rats as estimated by western blotting; **C**: Quantitative data of Daxx expression, results were normalized to β-actin. Data are the mean ± SE of three independent experiments. Control: normal food. HF: Added high fat to normal food. TC: Total cholesterol; TG: Triglyceride; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol. $^{a}P < 0.05$ *vs* control.

subjected to SDS-PAGE under reducing conditions, and proteins were then transferred to polyvinylidene difluoride membrane as described previously[13]. The membrane was blocked for 2 h at room temperature with a commercial blocking buffer from Life Technologies, Inc. The blots were incubated for 1 h at room temperature with the respective primary antibody (1:2000 dilution), which was followed by 1 h incubation with a secondary antibody (horseradish peroxidase-conjugated, 1:4000 dilution). Target proteins were visualized by a chemiluminescent assay (Amersham-Pharmacia Biotech).

Statistical analysis

The values are expressed as the mean ± SE. The correlation between cholesterol of serum and Daxx was analyzed by SPSS. Statistical analysis of the data was performed using student's *t* test or ANOVA. Values with $P < 0.05$ were considered statistically significant.

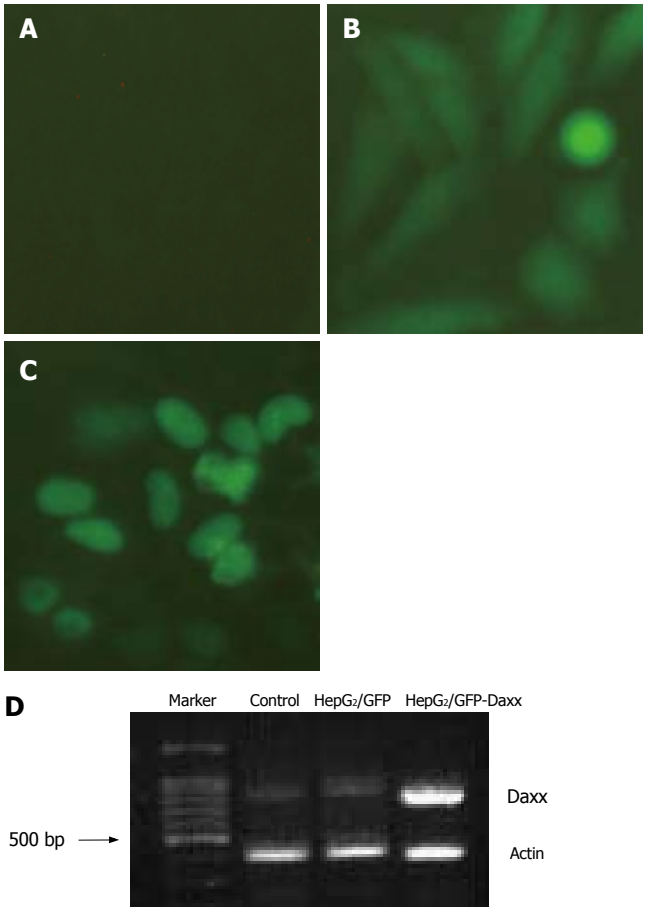

Figure 2 Daxx expression in HepG2 hepatocytes. Cultured HepG2 cells were untransfected (control, **A**) or transfected with pEGFP-C1-Daxx or pEGFP-C1 vectors (**B**, **C**). Images show the location and expression of Daxx in HepG2 cells, which were taken at 400 × magnitude. (**D**) RT-PCR of Daxx mRNA expression.

RESULTS

Correlation of Daxx and cholesterol

HFD feeding for 6 wk resulted in the development of hyperlipidemia in experimental rats, as shown in Figure 1A. Significant increases in TC (211%), TG (231%), HDL-C (197%), and LDL-C (246%) contents were observed in HFD-fed rats compared with those in control rats. At the same time, hepatic Daxx expression in the HFD-fed rats was decreased to one third of the control (Figure 1B and C). These data suggested that Daxx might have possible association with the change of blood lipid content as determined by correlation analysis. Further analysis revealed that there were negative correlations between Daxx and LDL-C (γ = -7.56, P = 0.018) and between Daxx and TC (γ = -9.07, P = 0.01), respectively.

Location and expression of Daxx in HepG2 hepatocytes

HepG2 cells were transfected with pEGFP-C1-Daxx or pEGFP-C1 plasmid. Daxx was mostly located in the nucleus of HepG2 cells (Figure 2A). Because the efficiency of transient-transfection was low, we screened out G418-resistant colonies. The majority of the colonies had fluorescence. PT-PCR analysis indicated that Daxx mRNA expression was significantly increased in HepG2/GFP-Daxx cells when compared with control or HepG2/GFP cells (Figure 2B).

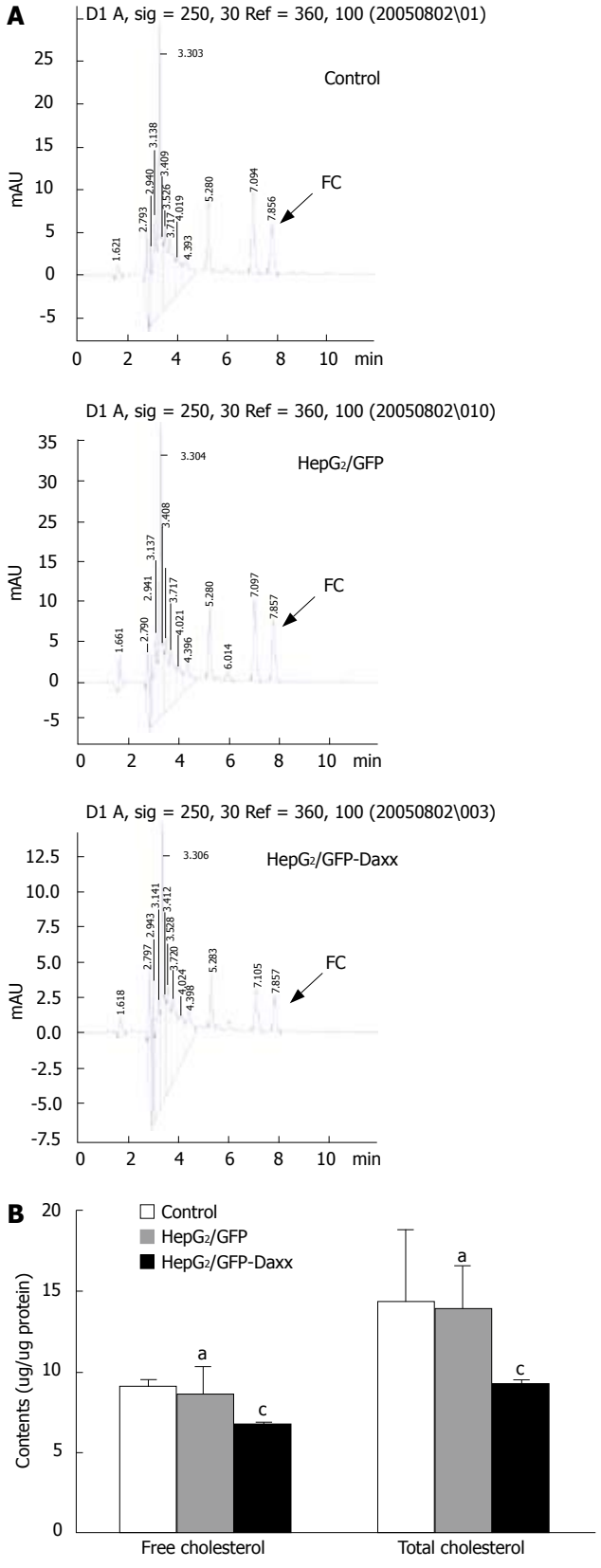

Figure 3 Effect of Daxx overexpression on cholesterol accumulation in HepG2 hepatocytes. **A**: Representative change of intracellular cholesterol levels in HepG2 cells, as determined by HPLC; **B**: The contents of free and total cholesterol in HepG2 cells. FC: Free cholesterol. [a]*P* > 0.05 *vs* control; [c]*P* < 0.05 *vs* control or HepG2/GFP cells.

Effect of Daxx on cholesterol content in HepG2 hepatocytes

Figure 3 shows the effect of Daxx on cholesterol concentration in HepG2 cells as analyzed by HPLC. The arrows show the area of apices which represent the contents of free cholesterol. The area of apices before the arrow represents the total cholesterol (Figure 3A). There was a significant decrease ($P < 0.05$) of the cholesterol concentration in HepG2/GFP-Daxx cells compared with other samples. The free cholesterol in HepG2/GFP-Daxx cells was 6.74 ± 0.13 (μg/μg protein), whereas those of control and HepG2/GFP cells were 9.21 ± 0.37 and 8.66 ± 1.72, respectively. The total cholesterol in HepG2/GFP-Daxx cells was lower than that of control or HepG2/GFP cells (9.28 ± 0.19 *vs* 14.36 ± 4.45 or 13.94 ± 2.62, both $P < 0.05$). The empty vectors did not show obvious effects on cholesterol concentrations in HepG2 cells (Figure 3B).

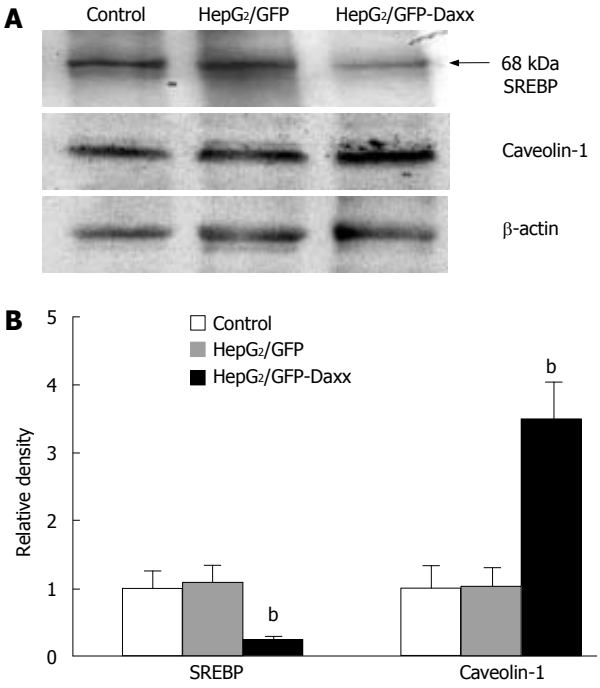

Figure 4 Effect of Daxx on the expression of SREBP and caveolin-1 proteins in HepG2 hepatocytes. **A**: Representative western blot data showing the effects of Daxx on SREBP and caveolin-1 proteins in HepG2 cells; **B**: Quantitative data of the Daxx effect on SREBP and caveolin-1 expression. Data are the mean ± SE of three independent experiments. [b]*P* < 0.01 *vs* control or HepG2/GFP cells.

Effect of Daxx on SREBP and caveolin-1 protein expression in HepG2 hepatocytes

Overexpression of Daxx in HepG2 cells significantly decreased the expression of activated-SREBP from 1 ± 0.23 to 0.21 ± 0.05. Likewise, caveolin-1 expression increased nearly 3.5 times compared to the control (1 ± 0.31 to 3.48 ± 0.56). The empty vectors did not show any effect on the proteins of HepG2 cells (Figure 4A and B).

DISCUSSION

In the animal experiment, we observed that expression of Daxx in hepatic tissues was negatively correlated with hyperlipidemia. The liver is a very important organ for maintaining the physical balance of lipids, and has many proteins (such as SREBP and caveolin) responsible for mediating cholesterol synthesis and excretion[14-16].

It has been reported that hepatic cells predominantly express Daxx[17], but the direct relation between Daxx and cholesterol still remains unclear. Recently, Daxx has been shown primarily to function as a transcriptional regulator[18-20]. These results indicate that Daxx could possibly affect cholesterol accumulation in hepatic cells.

In cultured $HepG_2$ cells, Daxx overexpression decreased the levels of FC and TC compared to those untransfected or transfected with GFP, which indicated that Daxx could affect cholesterol homeostasis of hepatic cells. HMG-CoA-reductase is a key enzyme of cholesterol synthesis and is regulated by sterol regulatory element binding proteins (SREBPs)[21,22]. SREBP-1 represents an important protein of the transcription regulator family (SREBP-1a, -1c, and -2) controlling lipid homeostasis in cells[23,24]. SREBP-1 are synthesized as 125-kDa inactive precursor proteins, and inserted into the membranes of the endoplasmic reticulum where they form tight complexes with SCAP. SREBP is proteolytically cleaved and activated by SCAP when the complex translocates to the Golgi apparatus[25]. The active 68-kDa SREBP fragment migrates to the nucleus and increases the transcription of sterol-responsive element (SRE) that contains many genes encoding lipogenic enzymes belonging to the pathways of cholesterol synthesis[26]. Daxx can inhibit AR transcriptional activity[6,7], which can down-regulates the activity of SCAP[9]. Thus, we checked the activity of SREBP-1 and detected that Daxx has the potential to decrease the expression of active SREBP-1. This also revealed that Daxx might mediate intracellular cholesterol accumulation by inhibiting cholesterol synthesis.

SREBP-1 represses caveolin expression by the SRE/SREBP pathway. In this case, SREBP inhibits *caveolin* gene transcription in contrast to its stimulating effect on other gene promoters[27,28]. Caveolin-1, a type of free cholesterol-binding protein, is another significant protein involved in cholesterol homeostasis[29]. Transfection of cells with full-length caveolin-1 cDNA resulted in the expression of morphologically authentic caveolae structure and FC efflux[30]. The expression of caveolin may represent a mechanism, by which FC excretion became facile[31]. The results of experiments showed that Daxx promoted the expression of caveolin-1. These findings show the possibility of Daxx mediating intracellular cholesterol accumulation presumably by increasing cholesterol excretion.

In conclusion, our results confirm that Daxx is likely to decrease the intracellular cholesterol accumulation by regulating cholesterol synthesis and excretion. One of the main future challenges will be the generation of suitable animal models to be used to dissect Daxx function in cholesterol homeostasis.

COMMENTS

Background

Death-associated protein (Daxx) could negatively modulate androgen receptor (AR) transcriptional activity. Androgens affect lipogenic gene expression not only in tumor cells but also in normal androgen target tissues *in vivo*. AR can directly upregulate sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP) by binding an androgen response element in intron 8 of SCAP gene. Activated SREBP can increase the mRNA and protein levels of genes involved in fatty acid, and cholesterol synthesis. These results indicate that Daxx could possibly regulate cellular cholesterol metabolism by the SREBP pathway.

Research frontiers

Hypercholesrerolemia is mainly a pathologic feature of cardiovascular diseases. The liver is a very important organ for maintaining the physical balance of cholesterol. In liver SREBPs are very important proteins which have been responsible for mediating cholesterol metabolism. Some studies have shown Daxx can regulate SREBP indirectly, but no evidences have suggested Daxx can affect cholesterol balance.

Innovations and breakthroughs

In the present study, we investigated the correlations between Daxx expression and cholesterol accumulation in liver cells. The findings herein show that overexpression of Daxx in $HepG_2$ cells may decrease intracellular cholesterol, which may be associated with inhibition of SREBP activity related to cholesterol synthesis and increase in caveolin-1 expression related to excretion.

Applications

Our study will provide academic value in finding a new function of Daxx, and experimental reference for clinical treatment of hypercholesterolemia.

Peer review

The authors demonstrated the inverse relationship between Daxx expression and cholesterol accumulation in hepatocytes. It appears that the effect of Daxx on cholesterol level in liver cells may be associated with inhibition of SREBP activity and an increase in caveolin-1 expression. The study was well performed and the data are clearly presented.

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BASIC RESEARCH

Experimental study on operative methods of pancreaticojejunostomy with reference to anastomotic patency and postoperative pancreatic exocrine function

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Abstract

AIM: To assess the patency of pancreaticoenterostomy and pancreatic exocrine function after three surgical methods.

METHODS: A pig model of pancreatic ductal dilation was made by ligating the main pancreatic duct. After 4 wk ligation, a total of 36 piglets were divided randomly into four groups. The piglets in the control group underwent laparotomy only; the others were treated by three anastomoses: (1) end-to-end pancreaticojejunostomy invagination (EEPJ); (2) end-to-side duct-to-mucosa sutured anastomosis (ESPJ); or (3) binding pancreaticojejunostomy (BPJ). Anastomotic patency was assessed after 8 wk by body weight gain, intrapancreatic ductal pressure, pancreatic exocrine function secretin test, pancreatography, and macroscopic and histologic features of the anastomotic site.

RESULTS: The EEPJ group had significantly slower weight gain than the ESPJ and BPJ groups on postoperative weeks 6 and 8 ($P < 0.05$). The animals in both the ESPJ and BPJ groups had a similar body weight gain. Intrapancreatic ductal pressure was similar in ESPJ and BPJ. However, pressure in EEPJ was significantly higher than that in ESPJ and BPJ ($P < 0.05$). All three functional parameters, the secretory volume, the flow rate of pancreatic juice, and bicarbonate concentration, were significantly higher in ESPJ and BPJ as compared to EEPJ ($P < 0.05$). However, the three parameters were similar in ESPJ and BPJ. Pancreatography performed after EEPJ revealed dilation and meandering of the main pancreatic duct, and the anastomotic site exhibited a variable degree of occlusion, and even blockage. Pancreatography of ESPJ and BPJ, however, showed normal ductal patency. Histopathology showed that the intestinal mucosa had fused with that of the pancreatic duct, with a gradual and continuous change from one to the other. For EEPJ, the portion of the pancreatic stump protruding into the jejunal lumen was largely replaced by cicatricial fibrous tissue.

CONCLUSION: A mucosa-to-mucosa pancreaticojejunostomy is the best choice for anastomotic patency when compared with EEPJ. BPJ can effectively maintain anastomotic patency and preserve pancreatic exocrine function as well as ESPJ.

Key words: Pancreaticojejunostomy; Animal model; anastomotic patency; Pancreatic exocrine function; Histopathology; Pancreatography

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INTRODUCTION

Pancreaticoduodenectomy is a well-established operation for resectable periampullary carcinoma. The anastomosis

between the remaining distal pancreas and the gastrointestinal tract after pancreaticoduodenectomy has, however, been the site of complications responsible for considerable morbidity and mortality. Pancreatic leakage is the most common and severe postoperative complication[1-5]. At present, most techniques have been directed toward the prevention of fistulas[3,6-11]. However, long-term anastomotic patency has not been of great importance in the past because of early cancer recurrence and death. Now that the results are improving in both cancer therapy and pancreatic transplantation, the issue of long-term anastomotic patency will grow in importance. Pancreaticoduodenectomy is an unusual gastrointestinal anastomosis in that a hollow muscular organ is being sewn to the side of a solid organ, with a small accentrically placed duct. Therefore, the anastomosis is prone to stenosis and obstruction because of the narrow lumen of the duct and fibrosis of the proximal pancreas after operation. At present, clinical and experimental studies have indicated a better patency rate for duct-to-mucosa anastomosis[12-20]. In theory, the optimum technique is to secure a leakproof anastomosis that will not subsequently produce stenosis and exocrine deficiency. The most important factor affecting the function of the remnant pancreas is the patency of the pancreaticojejunostomy and pancreaticogastrostomy. Data suggest that such stenosis and obstruction often results in eventual exocrine and endocrine dysfunction in animal models and patients[12-20].

Recently, Pen Shuyou *et al* have advocated the use of binding pancreaticojejunostomy (BPJ) after Whipple's resection to minimize pancreatic leakage. Clinical studies have revealed that the present technique is safe, simple and effective in fulfilling current demands on resectional pancreatic surgery[21-23]. So far, few studies appear to have investigated pathophysiological changes induced by BPJ following pancreaticoduodenectomy, particularly in relation to the anastomotic patency and function of the remnant pancreas. In the present study, we attempted to examine anastomotic patency and postoperative pancreatic exocrine function in piglets after three different methods of joining the body and tail of the pancreas to the gastrointestinal tract.

MATERIALS AND METHODS

Materials and animal model for pancreatic duct dilatation (first operation)

Thirty-six domestic piglets of both sexes, weighing 19-21 kg, were obtained from the Animal Center of Zhejiang University. All animals were treated humanely according to the national guidelines for the care of animals. The experiment was approved by the Animal Experiment Committee of the University of Zhejiang. The animals were fasted for 24 h before the experiment and allowed food and water *ad libitum* after the operation. The piglets underwent a preliminary sterile laparotomy under general anesthesia with 25 mg/kg sodium pentobarbital. The main pancreatic duct draining the body and tail was carefully separated and ligated with 1/0 silk thread, which produced total obstruction, and left the duodenal vascular arcade intact, as described by Pitkaranta *et al*[24]. This was then relieved by one of three methods at a second operation. For both operations, the animals were fasted the night before surgery. Intravenous cannulation and endotracheal intubation were performed after anesthetic induction. Animals received 1 L of normal saline during each procedure. Penicillin G benzathine in aqueous suspension (4 MU) was administered intramuscularly before the operation and continued the following day. The operation was performed under strict aseptic conditions. The controls underwent laparotomy only.

Pancreatic anastomosis (second operation)

Four weeks after ligation of the main duct, laparotomy was performed again. After resection of the pancreatic head (5 cm), the diameter of the duct at the cut surface was measured, and the pigs underwent sequentially one of three types of pancreatico-intestinal anastomosis, or were kept as controls. The management of the pancreatic stump was: (1) end-to-end pancreaticojejunostomy invagination (EEPJ) to a Roux-Y loop in nine piglets, using two layers (1/0 silk interrupted inner and outer layer); (2) end-to-side duct-to-mucosa sutured anastomosis to a Roux-Y loop (ESPJ) in nine piglets, using two layers (1/0 silk interrupted inner and outer layer), completed by a second layer between the pancreatic capsula and jejunal seromuscular wall; or (3) binding pancreaticojejunostomy (BPJ) in nine piglets. The surgical techniques have been described previously[21-23]. The main procedure was as follows. End-to-end duct-to-mucosa sutured (1/0 silk interrupted) anastomosis to a Roux-Y loop was performed, the pancreatic duct and jejunal mucosa were sutured, and the stitch was only inserted to the submucosal layer of the jejunal wall (Figure 1A). This procedure was slightly modified from those previously described. Finally, a piece of unabsorbable thread (7/0 silk) was used to bind circumferentially together the jejunal seromuscular sheath and pancreatic remnant (Figure 1B). The thread tie is just tight enough to allow the tip of a medium-sized hemostatic forceps to be passed underneath the ligature. No stent was used in any of the anastomoses. Roux-Y limbs of 45 cm were created using a standard side-to-side technique. After 2 d of water, the animals had free access to standard lab chow. Animals were kept in individual living quarters. Body weight was determined with a cage scale preoperatively and repeated at 2, 4, 6 and 8 wk during follow-up, until the third operation.

Measurement of intrapancreatic ductal pressure

Eight weeks after the second operation, laparotomy was again performed. Intrapancreatic ductal pressure was measured in all animals. As described by Bradley[25], a No 21 scalp vein needle (attached to a short length of connecting tube, a three-way stopcock, a manometer, and a 2-mL syringe, all filled with saline solution) was introduced into the duct of Wirsung directly through the surface of the pancreas. Fluid aspirated from the pancreatic duct into the syringe to confirm the position of the needle was meticulously replaced from the syringe before pressure measurement. The saline manometer was arbitrarily zeroed at the level of the anastomotic site. Fluctuation in pressure was noted with respiration in most animals. All pressures were recorded in the duct of Wirsung before operative pancreatography and surgical manipulation.

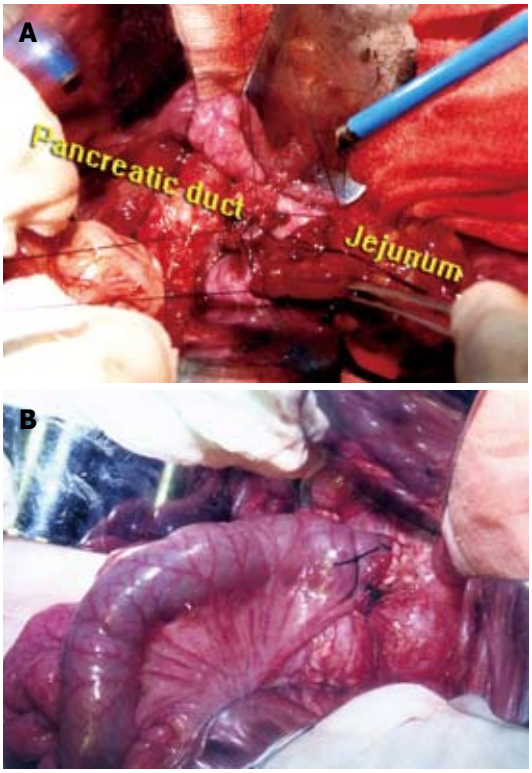

Figure 1 **A**: Pancreatic duct and jejunal mucosa are sutured, and stitch is only inserted to the submucosal layer of the jejunal wall; **B**: A piece of unabsorbable thread (7/0 silk) is used to bind together circumferentially the jejunal seromuscular sheath and pancreatic remnant.

Secretin test and minimal pressure required to obtain flow through the anastomosis

The jejunal wall was incised. The anastomotic site was grossly examined, and the status of the pancreatic drainage flow at the anastomosis was inspected by direct visual observation. Pancreatic exocrine function was evaluated by the secretin test. After 30 min basal secretion collection, secretin was administered (2 U/kg iv bolus; Sigma, St. Louis, MO, USA), and pancreatic juice at the anastomotic site was collected at intervals of 10 min for 60 min. Secretory volume, flow rate of pancreatic juice, and bicarbonate concentration were determined using standard laboratory techniques.

Pancreatographic investigation

After resection of the remnant pancreas and jejunum *en masse*, pancreatography was performed immediately by inserting a catheter into the main duct at the tail of the pancreas. The contrast medium (meglumine diatrizoate) was instilled from a height of 10 cm to equalize the pressure as uniformly as possible. The extracted pancreas was placed on an X-Omat TL film (Eastman Kodak, Rochester, NY, USA) for roentgenography at 300 mA, 26 kVp, 1.0 s and a film-focus distance of 88 cm. The maximum diameter of the main pancreatic duct was measured.

Macroscopic and histologic assessment

The pancreatic duct was examined for patency by cannulating the anastomosis distally, and testing to see if a catheter could be passed freely into the duct. Then, the anastomotic site and the long axis of the pancreatic duct of the body and tail were incised and examined macroscopically, and the specimens were fixed in 10% neutral formalin for 3 d. Sections containing the anastomotic site were prepared, embedded in paraffin, and stained with hematoxylin and eosin for histologic evaluation.

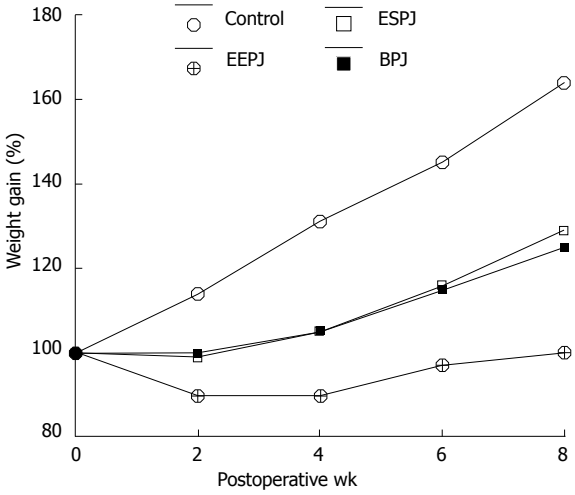

Figure 2 Percentage weight gain in pigs undergoing three types of pancreatoenteric anastomosis and in control pigs.

Statistical analysis

Data are presented as mean ± SD unless otherwise indicated. Statistical significance was assessed by analysis of variance or Student's *t* test where appropriate. Results were considered significant at $P < 0.05$.

RESULTS

Mean body weight changes

A percentage body weight change in relation to the preoperative value was determined for each animal. The postoperative weight gain for each group is shown in Figure 2. Duct-to-mucosa sutured anastomoses (ESPJ and BPJ) differed significantly from controls and EEPJ on postoperative week (POW) 2, 4, 6 and 8 ($P < 0.05$). The animals in the ESPJ and BPJ groups had a similar body weight gain, and these two types of anastomoses did not differ significantly from each other on POW 2, 4, 6 and 8 ($P > 0.05$). These mean body trends, however, suggested that the status of the anastomosis had some physiologic effect.

Intraperitoneal findings

All piglets recovered from the operation, and no fatality was encountered. Atrophy of the pancreatic parenchyma was shown in all animals in the three groups, and the main pancreatic duct at the cut surface of the pancreatic stump was markedly dilated at the end of POW 4 after the first operation (Figure 3, range 3.1-3.5 mm). At the end of POW 8 after the second operation, adhesion of the anastomotic portion to adjacent tissue was detected in all animals, the anastomotic site was healed completely, and no suture failure and intraperitoneal abscess formation

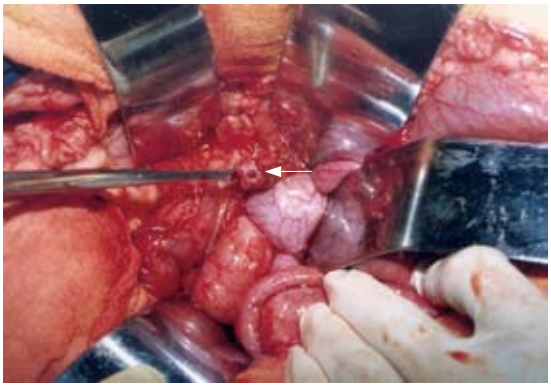

Figure 3 Duct at the cut surface of pancreatic stump was markedly dilated at the end of POW 4 after ligation of the main pancreatic duct.

Table 1 Average intraductal pressure in three types of pancreatic anastomosis and in controls

Group	*n*	Intraductal pressure (cm H_2O)
Control (normal)	9	13.24 ± 1.31
EEPJ	9	32.51 ± 2.19[a]
ESPJ	9	18.41 ± 1.42[a,c]
BPJ	9	19.13 ± 1.72[a,c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* EEPJ.

Table 2 Pancreatic secretion in three types of pancreatic anastomosis and in controls

Group	*n*	Secretory volume (mL/h)	Flow rate (mL/kg/30 min)	Bicarbonate concentration (mEq/L)
Control (normal)	9	137.56 ± 12.18	1.38 ± 0.13	133.56 ± 13.95
EEPJ	9	34.78 ± 4.82[a]	0.35 ± 0.10[a]	72.78 ± 10.58[a]
ESPJ	9	108.33 ± 10.65[a,c]	1.11 ± 0.15[a,c]	110.44 ± 12.52[a,c]
BPJ	9	101.23 ± 12.87[a,c]	1.05 ± 0.19[a,c]	112.67 ± 17.68[a,c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* EEPJ.

around the anastomotic site were observed in any animal. None of the piglets showed anastomotic complications.

Intrapancreatic duct pressure

Intrapancreatic duct pressure on POW 8 is shown in Table 1. The levels were similar in ESPJ and BPJ, and no significance was found between the two procedures ($P > 0.05$). However, pressure in EEPJ was significantly higher than that in ESPJ and BPJ ($P < 0.05$).

Pancreatic exocrine function assessed by the secretin test

The results of the secretin test for each group are summarized in Table 2. All three parameters, secretory volume, flow rate of pancreatic juice, and bicarbonate concentration, were significantly higher in ESPJ and BPJ compared to EEPJ ($P < 0.05$). However, the levels of the three parameters were similar in ESPJ and BPJ, with no significant difference between these two techniques ($P > 0.05$). These parameters suggested that the status of the anastomosis had some mechanical effect on pancreatic exocrine function.

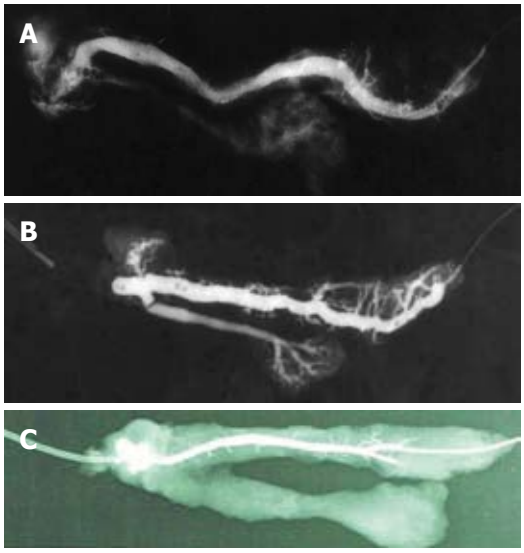

Figure 4 **A**: Anastomotic site was incompletely obstructed; **B**: Duct was blocked in the anastomotic region; **C**: Normal ductal patency without stenosis at the anastomotic site and without ductal dilatation proximal to it.

Pancreatography and maximum diameter of the main pancreatic duct

Pancreatography performed after EEPJ revealed dilation and meandering of the main pancreatic duct, together with indistinct depiction of the secondary and tertiary bifurcation of the duct, and the anastomotic site exhibited a variable degree of occlusion (Figure 4A). The duct was partially blocked in one animal and completely blocked in another in the anastomotic region (Figure 4B). Pancreatography of both ESPJ and BPJ, however, showed normal ductal patency without stenosis at the anastomotic site and without ductal dilatation and meandering of the main pancreatic duct, but distinct secondary and tertiary bifurcations of the ducts (Figure 4C). In one animal only, a slight dilatation of the anastomosed duct was detected in BPJ. Macroscopically, cicatricial narrowing of the main pancreatic duct was not observed, which suggested effective pancreatic juice drainage. The maximum diameter of the main pancreatic duct is shown in Table 3. Significant differences were found in both the ESPJ and BPJ groups in comparison with the EEPJ group ($P < 0.05$). Main pancreatic duct diameters showed no significant differences between ESPJ and BPJ ($P > 0.05$).

Macroscopic and histologic features of the anastomotic site

Macroscopically, in all specimens, there was firm union at the site of anastomosis, and the pancreas had an atrophic appearance. The specimens removed 8 wk after ESPJ and BPJ clearly showed complete mucosal continuity and good connective tissue union between the pancreatic duct and the jejunal lumen (Figure 5). The results were verified histologically, and microscopy showed that the intestinal mucosa had fused with that of the pancreatic duct, with gradual and continuous changes from one to the other (Figure 6). In contrast, in biopsies obtained in EEPJ, the portion of the pancreatic stump protruding into the jejunal lumen was largely replaced by cicatricial fibrous tissue in

Table 3 Maximum diameter of the main pancreatic duct

Group	*n*	Maximum diameter (mm)
Control	9	2.08 ± 0.33
EEPJ	9	6.13 ± 0.47[a]
ESPJ	9	2.98 ± 0.41[a,c]
BPJ	9	3.02 ± 0.45[a,c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* EEPJ.

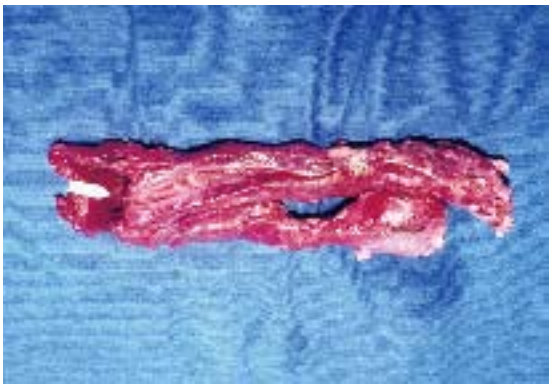

Figure 5 Longitudinal specimen reveals good connective tissue union between the pancreatic duct and the jejunal lumen.

which concentric fibrous bands with scattered mononuclear inflammatory cells were seen, and no intestinal mucosa was observed to cover the remnant cut surface.

DISCUSSION

Since Whipple's introduction of pancreaticoduodenectomy in 1935 for the treatment of cancer of the ampulla of Vater and the pancreatic head, it has become the treatment of choice in the management of periampullary cancer. However, the procedure incurs much criticism because of its high morbidity and mortality. Operative mortality has decreased dramatically over the past decades, due in part to improvement in operative technique and perioperative care[4,5,26]. Additionally, long-term survival has significantly improved and this has engendered a need to consider the importance of postoperative quality of life after resectional pancreatic surgery. The approaches involve the preservation of the exocrine and endocrine function by different techniques of anastomosis between the pancreatic stump and the digestive tract. Most of the proposed techniques are aimed at preventing leakage. Common procedures include EEPJ, ESPJ, and pancreatogastrostomy (which seems to be the least-performed procedure).

It is generally accepted that pancreatic consistency during Whipple resection is generally classified into one of two groups: normal, soft, and friable with non-dilated ducts, or firm and fibrotic with dilated ducts, with the former group having a high susceptibility to anastomotic leakage. The soft nature of the pancreatic remnant, combined with a relatively high pancreatic juice secretion rate through a non-dilated pancreatic duct, is thought to contribute to anastomotic leakage[27]. In many

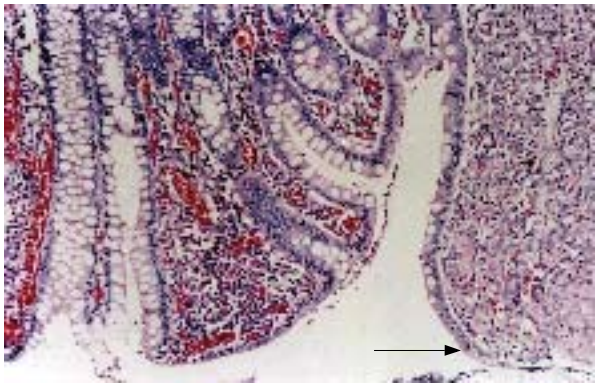

Figure 6 Section showing that the intestinal mucosa had fused with that of the pancreatic duct, with gradual and continuous changes from one to the other. Original magnification × 100.

patients with cancer and pancreatitis, the duct is dilated from obstruction, and the obstructed gland is fibrotic and easier to sew than when normal. Theoretically, the perfect approach is based on a simple general surgical principle: the absence of immediate leakage and the long-term patency of any intestinal pancreatic anastomosis. Previous studies have indicated the most important factor that influences the function of the remaining pancreas after pancreatoduodenectomy is the patency of pancreaticoenterostomy. There is a definite relationship between the degree of duct obstruction and the severity of the exocrine changes[12-20]. Therefore, preserving anastomotic patency may be essential for good remnant pancreatic function.

Tiscornia and Dreiling[28] ligated the main pancreatic duct and studied pancreatic secretion in 12 dogs with Thomas cannulas. After 7-46 d, the ligated segment of the main pancreatic duct was resected, and a primary duct-to-duct anastomosis was created to reestablish flow. Secretion was studied for up to 7 mo, and they found progressive improvement in bicarbonate and enzyme secretion, with eventual return to normal. Through clinical and experimental study, Tanaka and his colleagues[13] have revealed that the exocrine pancreas before pancreaticoduodenectomy was impaired due to obstructive pancreatitis, and that postoperative pancreatic function was well preserved at a level close to that in the control group, even after 50% resection of the pancreas, if pancreatic duct drainage was effectively performed. Several authors[12-20] have pointed out occlusion of the pancreatic duct after pancreaticoduodenectomy, which leads to an insufficiency in pancreatic exocrine and possibly endocrine function in later years. If this happens, the advantages of lower mortality and morbidity immediately after pancreaticoduodenectomy could be offset. It is conceivable that after the Whipple operation with extirpation of the sphincter of Oddi, the only outflow resistance encountered would be that due to stenosis at the site of pancreaticoenterostomy. At present, data obtained from both clinical and experimental studies have indicated that mucosa-to-mucosa pancreaticojejunostomy or pancreaticogastrostomy is the best choice for anastomotic patency[12-20].

Recently, the new method of BPJ has increasingly

been performed, especially in China, although other pancreaticojejunostomy techniques are also common. The clinical and experimental data have demonstrated that it is a safe, simple and efficient technique that avoids the primary complication of anastomatic leakage, whether the pancreatic remnant is soft or hard[21-23]. On the basis of these data, we have modified that approach slightly to test whether the patency of anastomosis and the function of the remnant pancreas of modified BPJ (mBPJ) is likely to be as good as, or better than that of the mucosa-mucosa anastomosis, for which the pancreatic remnant is hard and the pancreatic duct is dilated. To the best of our knowledge, no adequate studies appear to have been carried out.

The study of the patency of pancreaticoenterostomy and function of the remaining pancreas requires a suitable pancreatic duct dilatation model that results in an easier and more reliable experiment, although careful and meticulous anastomosis can be performed with microsurgical techniques. The digestive system of the pig closely resembles that of humans[24], therefore, we chose a duct-ligated model in piglets. This piglet model is reproducible and easy to perform, it produces changes in the pancreas analogous to those usually found in patients undergoing pancreatoduodenectomy, and the conclusion of the study is more convincing. Total obstruction of the main pancreatic duct in the experimental animals resulted in distention of pancreatic ducts and ductules.

In our previous study, we have demonstrated that there is a stronger anastomosis (bursting pressure and breaking strength) in BPJ than in EEPJ, and the anastomotic healing is better and more rapid, thus we conclude that BPJ is a safer and more reliable method than EEPJ, although no comparison has been made between BPJ and ESPJ[29]. However, the principle objectives of this study were to assess the patency of anastomosis, the exocrine function of the remnant pancreas, and the feasibility of mBPJ. The patency of anastomosis of mBPJ was the endpoint of the study.

In our present study, pancreatic duct patency without stenosis at the anastomotic site was confirmed directly by ductography and gross examination in all animals studied by ESPJ and BPJ. Similar patency was found in both ESPJ and BPJ. However, EEPJ revealed dilation and meandering of the main pancreatic duct, which seemed to be caused by pancreatic juice retention, due to poor drainage of pancreatic juice in the anastomosed pancreas. The degree of dilatation of the main pancreatic duct seems to correspond to the likelihood of anastomotic stenosis of pancreaticoenterostomy. The body weight gain (which is positively related to the pancreatic exocrine secretion[30]), secretin test, and intrapancreatic ductal pressure are in agreement with the functional status of the pancreatic duct and anastomotic site. Jejunal mucosa is in continuity with the pancreatic duct at the anastomotic site in ESPJ and BPJ. Consequently, we were able to obtain a very secure patent anastomosis in the piglet model. Our data were in accordance with those of previous studies that reported better patency with mucosa-mucosa anastomosis compared with EEPJ. According to the results of our study, and taking into consideration the advantage of preventing anastomotic breakdown or pancreatic leakage and maintaining patency of the pancreatic duct by BPJ, we believe that the procedure will result in a more anatomically secure anastomosis than that with the standard method, and should decrease the morbidity and mortality of the operation, thus increasing its acceptability as the operation of choice for Whipple resection.

COMMENTS

Background

Resectional pancreatic surgery is carried out with increasing frequency for both malignant and benign diseases. However, no adequate studies appear to have been made of the pathophysiological changes following pancreaticoduodenectomy, particularly of anastomotic patency and function of the remnant pancreas. The most important factor affecting the function of the remnant pancreas is the patency of the pancreaticoenterotomy. In the present study, we attempted to examine anastomotic patency and postoperative pancreatic exocrine function in piglets after three different anastomotic methods using end-to-end pancreaticojejunostomy invagination (EEPJ), end-to-side duct-to-mucosa sutured anastomosis (ESPJ) and binding pancreaticojejunostomy (BPJ) (a new method).

Research frontiers

There is little agreement about the best technique for pancreatic anastomosis of the gastrointestinal tract. The optimum technique is to secure a leakproof anastomosis that will not subsequently produce stenosis and exocrine deficiency. However, few clinical and experimental studies have focused on the postoperative pancreatic exocrine function. The main findings of this study were that the animals undergoing ESPJ and BPJ were much better than those undergoing EEJP, using several parameters of measurement, weight gain, intraductal pressure, secretory volume and flow of pancreatic juice, ductal patency and absence of duct dilatation.

Innovations and breakthroughs

As a new method of anastomosis, BPJ has been proven to be a safe, simple and efficient technique that avoids the primary complication of anastomatic leakage clinically and experimentally. The authors also confirmed that BPJ may effectively maintain anastomotic patency and preserve pancreatic exocrine function as well as ESPJ, and a mucosa-to-mucosa pancreaticojejunostomy is the best choice for anastomotic patency, when compared with EEPJ.

Applications

Our data were in accordance with those of previous studies that have reported better patency with mucosa-mucosa anastomosis when compared with EEPJ. According to our results, and taking into consideration the advantage of preventing anastomotic breakdown or pancreatic leakage and maintaining patency of the pancreatic duct by BPJ, we believe that this procedure will result in a more anatomically secure anastomosis than that with the standard method, and should decrease the morbidity and mortality of the operation, thus increasing its acceptability as the operation of choice for Whipple resection.

Terminology

BPJ, a new method introduced to pancreatic surgery by Pen Shuyou *et al*, has increasingly been performed, especially in China, although other pancreaticojejunostomy techniques are also common. Clinical studies have revealed that the present technique is safe, simple and effective in fulfilling current demands on resectional pancreatic surgery. However, the anastomotic patency and residual exocrine pancreatic function after BPJ continues to require adequate evaluation.

Peer review

The authors are to be commended on their excellent piece of research. There have been few clinical and experimental studies with a focus on the pathophysiological changes of pancreaticoenterotomy, particularly of anastomotic patency and function of the remnant pancreas. This is an interesting study, well designed and well performed. The discussions are to the point and not overstated. The authors' findings certainly merit attention.

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CLINICAL RESEARCH

Management of gastric fundal varices without gastro-renal shunt in 15 patients

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Abstract

AIM: To examine the portal hemodynamics of gastric fundal varices (GV) without gastro-renal shunt (GRS), and to retrospectively investigate the effects of various kinds of treatment on eradication.

METHODS: Ninety-four liver cirrhosis patients at high-risk of GV were treated in our hospital and enrolled in this study. We retrospectively examined their characteristics, liver function, and portal hemodynamics of GV. We performed balloon-occluded retrograde transvenous obliteration (BRTO) at first. If it was not technically possible to perform BRTO, endoscopic injection sclerotherapy using α-cyanoacrylate glue (CA) or percutaneous transhepatic obliteration (PTO) was performed.

RESULTS: Among the 94 patients, a GRS was present in 79 (84.0%), and absent in the remaining 15 (16.0%). The subphrenic vein was connected to the inferior vena cava as the drainage vein in 13 (86.7%) out of the 15 cases without GRS. We performed BRTO in 6 patients, CA in 4 patients and PTO in 5 patients. The eradication rate was 100% for each procedure, but the rate of early recurrence within 6 mo was 16.7% for BRTO, 50.0% for CA and 40.0% for PTO, respectively.

CONCLUSION: We should examine the hemodynamics before treatment of GV irrespective of the existence of GRS. If this hemodynamic examination reveals that the drainage vein connects directly to the inferior vena cava in GV without GRS, BRTO may be an effective treatment for GV with GRS.

Key words: Gastric fundal varices; Gastro-renal shunt; Balloon-occluded retrograde transvenous obliteration

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INTRODUCTION

Rupture of esophagogastric varices is one of the most severe complications of portal hypertension in patients with liver cirrhosis. Gastric varices bleed less frequently than esophageal varices, but bleeding from gastric fundal varices (GV) tends to be more severe and is associated with a high mortality rate[1,2]. Effective management of such bleeding is essential. Endoscopic injection sclerotherapy and endoscopic variceal ligation are currently the mainstay of treatment for esophageal varices. Most cases of GV cannot be treated effectively with endoscopic injection sclerotherapy alone[3-5] because of the rapid blood flow within their vessels with a large diameter. Endoscopic injection of n-butyl-2-cyanoacrylate or isobutyl-2-cyanoacrylate is used as the standard first-line treatment for bleeding GV[6] in the Western countries. However, the reported severe complications related to embolization of other organs have raised concerns for the safety of injection of tissue adhesive agents[7-11]. Transjugular intrahepatic portosystemic shunt (TIPS) placement, one of the interventional radiological techniques, is currently the second-line treatment for bleeding esophagogastric varices[6,12] and can effectively control GV bleeding[13-15]. However, it was reported that this treatment is associated with a lower success rate for GV than for esophageal varices[16-18].

Recently, some newer interventional radiological techniques have been used to treat GV with good results. Balloon-occluded retrograde transvenous obliteration (BRTO) is a very useful treatment for GV in terms of efficacy, safety, and degree of invasiveness[19-29], and the recurrence rate of GV has been also reported to be 0%-10%[23-29] after BRTO, which shows excellent long-term results. Prophylactic treatment with BRTO can effectively prevent GV rupture, and improve patient survival[28]. Ninoi *et al*[29] showed that GV bleeding is better controlled with BRTO than with TIPS. Therefore, not only radiologists but also hepatologists and gastroenterologists in Japan have considered BRTO the standard first-line treatment for GV in place of endoscopic variceal obturation therapy or TIPS. However, it is generally considered that BRTO cannot be used in the treatment of GV without GRS because BRTO is performed through GRS, which exists in almost 85% of GV[7]. It is very crucial to investigate whether BRTO can be feasible for GV without GRS and the effects of BRTO compared with other methods.

The portal hemodynamics of GV without GRS is not well known, and there is no well-established treatment for the eradication of these GV. This study was to examine the portal hemodynamics of GV without GRS compared with those with GRS, and to retrospectively investigate the effects of BRTO compared with some kinds of treatment on the eradication of these varices.

MATERIALS AND METHODS

Ninety-four liver cirrhosis patients at high-risk of GV were treated in our hospital between February 1996 and June 2002 and enrolled in this study. All patients had GV with acute bleeding or were at high-risk of GV. GV were evaluated based on esophagogastroduodenoscopy (EGD) criteria according to the system adopted in Japan[30,31]. In brief, the location of gastric varices (Lg) was classified as being adjacent to the cardiac ring (Lg-c), separated from the cardiac ring (Lg-f), or continuing from the Lg-f to the gastric fundus (Lg-cf). Varices were further classified as straight and small caliber varices (F1), beaded varices (F2), or tumor-shaped varices (F3). We categorized GV as a high risk according to the criteria reported by Kim *et al*[32], who found that a diameter of at least 5 mm and the presence of red spots are independent factors that predict a high-risk of variceal bleeding. All patients were evaluated for variceal hemodynamics with color Doppler endoscopic ultrasonography (CD-EUS) and multidimensional computed tomography (MDCT) or CD-EUS and magnetic resonance angiography (MRA). Of the 94 patients, 23 were urgent or elective cases with episodes of variceal bleeding, and 71 were prophylactic cases. We retrospectively examined their characteristics, liver function, and portal hemodynamics of GV. CD-EUS (FG-32UA, Pentax Co., Ltd., EUB-555 US scanner, Hitachi Co., Ltd., Tokyo, Japan) was performed in all patients to measure the diameters of variceal vessels as well as blood flow velocity before treatment. Blood flow was calculated from blood vessel diameter and blood flow velocity. All patients underwent MDCT or MRA to detect the supply and drainage vein and to determine whether they had a gastro-renal shunt. The patients underwent repeated CD-EUS within 1 wk after treatment to assess the sclerosing effect. The efficiency of each treatment was confirmed by the rate of disappearance of varices. EGD was done every 3-6 mo after treatment to evaluate the status of GV. The recurrence of GV was defined as detection of appearance of high-risk signs (as described above) or variceal bleeding. The follow-up period for recurrence was calculated as days from the date of treatment until the first date when EGD revealed recurrence.

Regarding the selection of the method for treatment, BRTO is the first-line treatment for GV with GRS in our hospital because it is generally considered the first-line treatment in Japan[19-29]. Therefore, in cases where it was possible, we performed BRTO even for varices without GRS. If it was not technically possible to perform BRTO, endoscopic injection sclerotherapy using α-cyanoacrylate glue (CA) or percutaneous transhepatic obliteration (PTO) was used.

BRTO procedure

BRTO is a method for treating GV by injection of a sclerosant after the main drainage vein of GV is blocked angiographically to stagnate blood flow. In general, a balloon catheter is inserted *via* the right femoral vein and wedged into the left adrenal vein. After the balloon is inflated, the gastro-renal shunt is visualized with the contrast agent iopamidol to judge whether shunt occlusion has been achieved. In case of GV without GRS, if the drainage vein is connected directly to the inferior vena cava, BRTO could be performed after an occlusive balloon catheter is placed in the drainage vein through the right femoral vein (Figure 1A and B). If retention of contrast agent is insufficient in GV because of other collateral veins, metallic coils are placed in the collateral veins to reduce the blood flow. In our study, after venography showed sufficient retention of contrast agent in GV, 5% solution of ethanolamine oleate with iopamidol (EOI) was continuously injected through the catheter in the drainage vein until the varices were sufficiently filled with the sclerosant. Thereafter, the catheter was left in place for 24 h to allow sclerosis to occur within the gastric fundal varices.

Modified PTO procedure with injection of sclerosant

The modified PTO using sclerosant and metallic coils[29,33] can embolize GV more selectively than original PTO[34]. In our study, percutaneous transhepatic portography was performed, and the supply and drainage veins of the GV were identified on portography. After metallic coils were placed in the supply vein to reduce blood flow in the GV, EOI was injected through the catheter until the gastric fundal varices were sufficiently filled with the sclerosant.

CA procedure

CA is a method for treating GV by injection of a sclerosant endoscopically. In our study, a sclerotherapy injector, with a 20 gauge needle, was used for variceal injection. The GV were endoscopically punctured and

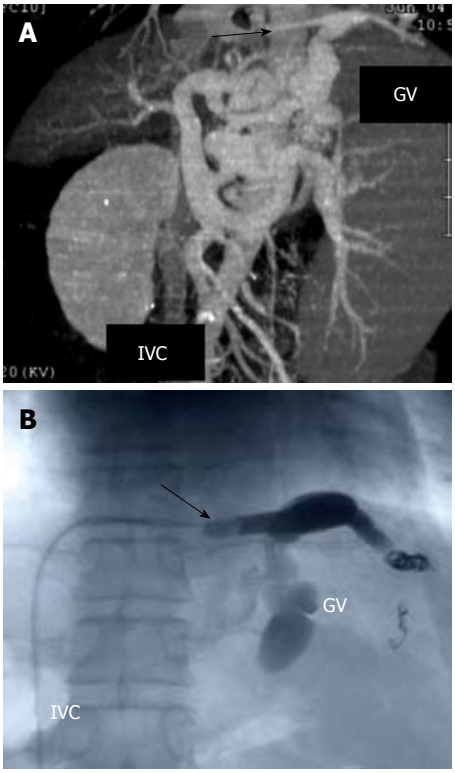

Figure 1 Multidimensional computed tomography (MDCT) revealing the varices supplied by the left gastric vein and drained into the subphrenic vein (arrow) which is connected to the inferior vena cava (IVC) (**A**), and fluoroscopic image of BRTO showing placement of the occlusive balloon catheter in the subphrenic vein (arrow) (**B**). After the blood vessels from the GV except for the main drainage vein were blocked with coils, 5% solution of ethanolamine oleate with iopamidol (EOI) was injected into the varices.

injected with 2.5 mL of a lipiodol-α-cyanoacrylate mixture (64% α-cyanoacrylate) in one shot, and injection was stopped when the varices were filled sufficiently after several injections.

Statistical analysis

Values are presented as mean ± SD, or as percentages. The Mann-Whitney *U* test was used to assess differences in age, sex, form, location, diameter of variceal vessels, blood flow velocity, blood flow volume, and liver function tests. Categorical variables were analyzed by the chi square test, with Yates' correction for continuity where appropriate, or by the Fisher's exact test. The Kaplan-Meier method was used to calculate the recurrence rate for GV. Differences between the groups were compared by means of the log-rank test. Statistical software (SPSS 10.0J, SPSS Japan Inc., Tokyo, Japan) was used in statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Ninety-four patients (60 men, 34 women; mean age 62.3 ± 9.7 years, range 33-80 years) underwent hemodynamic evaluation of GV with CD-EUS and MDCT or CD-EUS and MRA. Among the 94 patients, hepatic function was classified as class A in 53, class B in 36 and class C in 5 by the Child-Pugh classification. The location of GV was at Lg-f in 16 patients and at Lg-cf in 78 patients. The form of gastric varices was F2 in 32 patients and F3 in 62 patients. There were 14 patients who had red color spots on the GV. Among the 94 patients, a GRS was present in 79 (84.0%), and absent in the remaining 15 (16.0%) (Table 1).

Table 1 Clinical characteristics of patients and profile of varices in GRS (+) and GRS (-) groups *n* (%)

	GRS (+) (*n* = 79)	GRS (-) (*n* = 15)	*P* value
Age: yr (range)	61.9 ± 10 (42-80)	61.5 ± 10 (44-76)	NS
Gender (Male:Female)	1:0.58	1:0.5	NS
Child-Pugh classification			
A	47 (59.5)	6 (40)	NS
B	28 (35.4)	8 (53.3)	NS
C	4 (5.1)	1 (6.7)	NS
Endoscopic featres of GV			
Form			
F2	27 (34.1)	5 (33.3)	NS
F3	52 (65.8)	10 (66.7)	NS
Location			
Lgf	12 (15.2)	4 (26.7)	NS
Lgcf	67 (84.8)	11 (73.3)	NS
Presence of red color spots	12 (15.2)	2 (13.3)	NS
History of GV bleeding	20 (25.3)	3 (20)	NS
Finding of CD-EUS			
Blood vessel diameter (mm)	9.5 ± 3.4	6.7 ± 2.8[a]	0.009
Blood flow velocity (cm/s)	16.5 ± 4.3	14.5 ± 3.5	NS
Blood flow volume (cm^3/s)	50.8 ± 45.0	26.1 ± 25.7[a]	0.043

NS: Not significant. GV: Gastric fundal varices; GRS: Gastro-renal shunt; CD-EUS: Color doppler endoscopic ultrasonography. Values for age and findings of CD- EUS are the median. [a]$P < 0.05$ *vs* GRS (+) group.

Regarding the hemodynamics of GV without GRS, there were a number of different supply veins, including the left gastric vein (21.7%), the short gastric vein (18.8%), and the retrogastric vein (59.4%). However, among the 15 cases without GRS, the subphrenic vein was connected to the inferior vena cava as the drainage vein in 13 cases (86.7%) out of the 15 cases without GRS (Table 2). Between the GRS (+) and GRS (-) groups, there were no significant differences in age, sex, form and location of the varices, presence of red-colored spots, velocity of blood flow in the GV, or in Child-Pugh classification. The diameter of varices and blood flow volume were higher in the GRS (+) group than in the GRS (-) group ($P = 0.009$, $P = 0.043$) (Table 1). Regarding the treatment of GV without GRS, we performed BRTO in 6 patients, CA in 4 patients and PTO in 5 patients, without severe complications (Table 2). The GV eradication rate in the GRS (-) group for each procedure was 100%.

The rate of early recurrence, within 6 mo, was 16.7% for BRTO, 50.0% for CA and 40.0% for PTO, respectively, in the GRS (-) group (Table 3). When the main drainage vein was found to be connected to the inferior vena cava, BRTO was the most effective treatment even for GV without GRS. No severe complication occurred in each method except for slight abdominal discomfort or hematuria. Most of the patients treated with BRTO or PTO revealed hematuria caused by sclerosant, but it was recovered when haptoglobin was used without renal damage (haptoglobin was used prophylactically in all cases treated with BRTO or PTO).

Table 2 Gastric fundal varices without gastro-renal shunt

Age	Sex	Form	Diameter (mm)	Verocity (cm/s)	Supplying vein	Drainage vein	Treatment
75	M	LgcfF2	6.8	9.3	Rgv, Sgv	Spv	CA
45	M	LgcF2	8.8	15.0	Lgv, Rgv	Spv	CA
69	F	LgcfF3	12.0	20.1	Lgv	Pev	CA
52	M	LgcfF2	6.1	14.3	Lgv, Sgv	Spv	CA
67	M	LgfF3	4.5	15.0	Lgv, Rgv	Spv, Pcv	PTO
66	M	LgcfF3	4.7	18.5	Lgv, Rgv, Sgv	Pcv	PTO
64	M	LgcF3	4.8	18.1	Rgv	Spv	PTO
65	M	LgcfF3	7.2	10.5	Rgv	Spv	PTO
76	F	LgcF2	3.8	12.0	Lgv	Spv, Pev	PTO
44	F	LgcfF3	8.0	13.0	Rgv, Lgv	Spv, Pcv	BRTO
66	F	LgfF3	11.0	17.7	Rgv	Spv	BRTO
54	M	LgfF3	10.0	17.7	Lgv	Spv	BRTO
52	M	LgfF3	6.0	15.6	Lgv, Rgv, Sgv	Spv, Pcv	BRTO
64	M	LgcfF3	4.4	12.4	Sgv	Spv	BRTO
63	M	LgcF2	2.0	8.9	Lgv	Spv	BRTO

CA: Endoscopic injection sclerotherapy using α-cyanoacrylate glue; Rgv: Retro gastric vein; Sgv: Short gastric vein; Lgv: Left gastric vein; Spv: Subphrenic vein; Pev: Para esophageal vein; Pcv: Peri-cardial vein; BRTO: Balloon occluded retrograde transvenous obliteration; PTO: Percutaneous transhepatic obliteration.

Table 3 Treatment methods and recurrence rate of varices in GRS (-) group *n* (%)

GRS (-)	*n* = 15	*P* value
Treatment		
BRTO	6 (40)	NS
PTO	5 (33)	NS
CA	4 (27)	NS
Early recurrence rate within 6 mo		
BRTO	1 (16.7)	
PTO	2 (40)	
CA	2 (50)	

BRTO: Balloon-occluded retrograde transvenous obliteration; PTO: Percutaneous transhepatic obliteration; CA: Endoscopic injection sclerotherapy using α-cyanoacrylate glue; GRS: Gastro-renal shunt.

DISCUSSION

The present study is the first clinical study examining the portal hemodynamics of GV without GRS compared with those with GRS and the effects of some kinds of treatment for high-risk GV without GRS. The hemodynamic patterns in patients with GV are distinctly different from those in patients with esophageal varices[6,35]. Solitary GV are more frequently supplied by the short and posterior gastric veins, and drain to the inferior vena cava through GRS. However, this shunt is absent in around 15% of patients with GV[25]. The present study revealed that in 13 (87%) of 15 cases without GRS, the subphrenic vein was connected to the inferior vena cava as a drainage vein. Although the varices in the GRS (-) group had thinner veins and a lower blood-flow volume than those in the GRS (+) group, the hemodynamics in the GRS (-) group was more complicated than that in the GRS (+) group, suggesting that it is necessary to examine hemodynamics before treatment, so that the most suitable treatment can be selected.

There is no established treatment for the eradication of GV in these patients without GRS. Our results show that the effect of BRTO on GV without GRS was excellent. The eradication rate of GV without GRS for each procedure (BRTO, PTO, and CA) was high, but BRTO was superior to CA and PTO in early recurrence rate within 6 mo (16.7% for BRTO, 50.0% for CA and 40.0% for PTO). In this respect, no randomized controlled trials comparing BRTO with CA or PTO are available. Regarding the long-term efficacy of CA, GV rebleeding occurs in 23%-50% of patients with most of them occurring in the first year[6]. In our hospital, the recurrence rates at 1, 3, and 5 years of GV with GRS are 2.7%, 2.7%, and 2.7%, respectively, for BRTO in 78 patients[27], 4%, 41.5%, and 53.2%, respectively, for CA in 38 patients, and 28.6%, 42.9%, and 61.9%, respectively, for PTO in 13 patients (data not shown). These results suggest that recurrence rates of GV after BRTO are lower than those of GV after other procedures, showing the excellent long-term effect of BRTO. We speculate that the reason why CA and PTO are associated with a higher recurrence rate than BRTO may be due to insufficient injection of the occlusive substances into the gastric varices and the drainage vein with the PTO and CA procedures, leading to recanalization of the GV. The results of this study suggest that BRTO is an effective treatment for GV without GRS as well as for GV with GRS.

When examination of the hemodynamics reveals that the main drainage vein is connected directly to the inferior vena cava, BRTO is indicated for GV not only with GRS but also without GRS if a catheter can be inserted into the main drainage vein. The purpose of this method is to inject the sclerosant from the drainage vein, so the efficacy of treatment may depend on the sufficient retention of the sclerosant rather than on the approach route. Therefore there is no difference in efficacy depending on the presence or absence of GRS. The most important factor for performing BRTO is the presence of the major shunt directly connected to the inferior vena cava. If the drainage vein is too thin to insert the catheter or not connected to the inferior vena cava, it is impossible to perform BRTO.

Various techniques are now available for the treatment of GV (Figure 2). PTO is used for the treatment of ruptured varices, but the use of this method has decreased following the development of endoscopic treatment. The modified PTO using sclerosant and metallic coils[29,33] can embolize GV more selectively than the original PTO[34]. When the drainage vein is not connected to the inferior vena cava, the modified PTO might be a good option for the treatment of GV without GRS, and some good results have been reported[29,33]. However, this method is not used outside Japan as BRTO. TIPS placement is currently the second-line treatment for bleeding esophagogastric varices[6,12] and can effectively control GV bleeding[13-15]. However, it was reported that its rate (25%-63%) is lower for GV than for esophageal varices[14-16]. Because patients with GV are reported to have a lower portocaval pressure gradient[35], they have extensive spontaneous portosystemic shunts and respond poorly to TIPS. Ninoi *et al*[29] reported that transcatheter sclerotherapy using BRTO

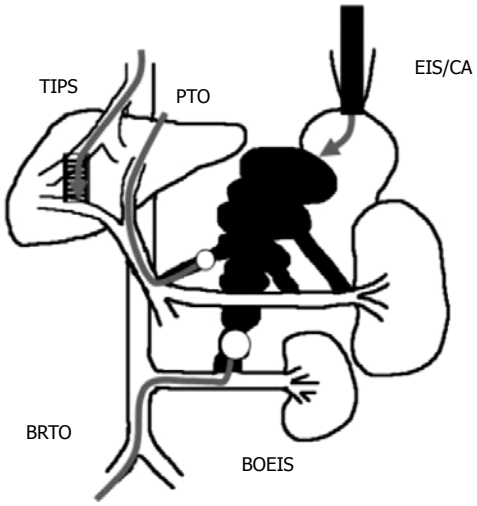

Figure 2 Treatment of gastric fundal varices with balloon-occluded retrograde transvenous obliteration (BRTO), percutaneous transhepatic obliteration (PTO), balloon-occluded endoscopic injection sclerotherapy (BO-EIS), endoscopic injection sclerotherapy using α-cyanoacryrate glue (CA), transjugular intrahepatic portosystemic shunt (TIPS).

and modified PTO can control GV bleeding better than TIPS[29]. However, larger prospective studies need to be performed. We reported that balloon-occluded endoscopic injection sclerotherapy (BOEIS) has an advantage in that it appears to be applicable to patients with GV without GRS[36]. However, BOEIS needs both interventional and endoscopic methods, and is more invasive than other methods. BOEIS is limited only to cases for which other methods are not suitable.

In conclusion, we should examine the hemodynamics before treatment of GV irrespective of the existence of GRS. If this hemodynamic examination reveals that the drainage vein is connected directly to the inferior vena cava in GV without GRS, BRTO might be an effective treatment for GV with GRS as well.

COMMENTS

Background

Recently, a newer interventional radiological technique has been used to treat gastric fundal varices (GV) with good results. Balloon-occluded retrograde transvenous obliteration (BRTO) is a very useful treatment for GV in terms of efficacy, safety, and degree of invasiveness.

Research frontiers

The present study is the first clinical study examining the portal hemodynamics of GV without gastro-renal shunt (GRS) compared to those with GRS and the effects of some kinds of treatment for high-risk GV without GRS.

Innovations and breakthroughs

BRTO is a very useful treatment for GV. However, it is generally considered that BRTO cannot be used in the treatment of GV without GRS. The results of this study suggest that BRTO may be an effective treatment for GV without GRS as well as for GV with GRS.

Applications

When hemodynamic examination reveals that the drainage vein is connected directly to the inferior vena cava in GV without GRS, BRTO might be an effective treatment for GV with GRS as well.

Terminology

BRTO: A method for treating GV by injecting a sclerosant after the main draining vein of GV is blocked angiographically to stagnate for blood flow.

Peer review

In this study, the authors described the hemodynamics and response to different treatments modalities for gastric varices without GRS. The authors support the use of BTRO as the first line treatment for patients in which the drainage vein is connected directly to the inferior vena cava.

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CLINICAL RESEARCH

Effectiveness and safety of herbal medicines in the treatment of irritable bowel syndrome: A systematic review

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Abstract

AIM: To explore the efficacy and safety of herbal medicines (HM) in the treatment of irritable bowel syndrome (IBS).

METHODS: A computer-based as well as manual literature search was performed. We reviewed randomized controlled trials on the treatment of IBS with and without HM.

RESULTS: A total of 22 studies with 25 HMs met the inclusion criteria. Four of these studies were of good quality, while the remaining 18 studies involving 17 Chinese herbal medicine (CHM) formulas were of poor quality. Eight of these reports using 9 HMs showed global improvement of IBS symptoms, 4 studies with 3 HMs were efficacious in diarrhea-predominant IBS, and 2 studies with 2 HMs showed improvement in constipation-predominant IBS. Out of a total of 1279 patients, 15 adverse events in 47 subjects were reported with HM. No serious adverse events or abnormal laboratory tests were observed. The incidence of the adverse events was low (2.97%; 95% CI: 2.04%-3.90%).

CONCLUSION: Herbal medicines have therapeutic benefit in IBS, and adverse events are seldom reported in literature. Nevertheless, herbal medicines should be used with caution. It is necessary to conduct rigorous, well-designed clinical trials to evaluate their effectiveness and safety in the treatment of IBS.

Key words: Irritable bowel syndrome; Herbal medicine; Systematic review

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INTRODUCTION

Irritable bowel syndrome (IBS) is associated with a variable combination of chronic or recurrent symptoms such as abdominal pain, bloating, constipation and diarrhea. There is generally no structural or biochemical abnormality detected by conventional laboratory tests. IBS is one of the most common functional gastrointestinal disorders accounting for 3% of all primary consultations[1]. In western countries, the prevalence of IBS is around 10%, depending upon the definition used[2]. Moreover, there is increasing prevalence of IBS in the newly developed Asian countries[3]. The potential etiological factors include stress, anxiety, visceral hypersensitivity, altered bowel motility, neurotransmitter imbalances, and inflammation.

Herbal medicines have been used in Asia for a long time. An increasing number of IBS patients are beginning to receive complementary and alternative medicines in the West, most frequently herbal remedies (43%)[4]. Patients may seek HM for symptomatic relief when conventional medicines (CM) are unsuccessful. In such situations, an important question is whether herbal medicines are effective and safe for IBS patients. In the present study, we systematically reviewed the literature and evaluated the effects of HM as well as their potential adverse events in patients with irritable bowel syndrome.

MATERIALS AND METHODS

Search strategy

We carried out a literature search using MEDLINE (1966-2005), EMBASE (1980-2005), Cochrane Database (1992-2005), TCMLARS Database (1984-2005), CJA Full-Text Database (1994-2005), and Chongqing VIP Database (1989-2005) for relevant randomized controlled clinical trials, meta-analysis and systematic reviews published in all languages until October 2005. We used MeSH terms including 'irritable bowel syndrome, functional colonic

disease, drugs, Chinese medicines, traditional medicines, herbal medicines, alternative medicines, complement medicines, plant, oriental traditional medicine' for the database search.

In addition, a hand search of reference lists, review articles, editorials and abstracts from major meetings was also conducted to supplement the electronic search. We included articles published in all languages. Titles and abstracts of all potentially relevant studies were screened before retrieval of the full articles. However, if the title and the abstract were ambiguous, the full articles were scrutinized. Two independent reviewers (J.S and H-X. L) participated in the literature search. Any disagreements were resolved by discussions in order to reach a consensus.

Inclusion and exclusion criteria

The following inclusion criteria were used: (1) Diagnostic criteria used for IBS were ROME I [5] or ROME Ⅱ [6] criteria or the 1986 National symposium on chronic diarrhea (Chengdu China) criteria[7]; (2) Study design with randomized controlled clinical trials, irrespective of blinding; (3) Studies using HM alone for treating IBS in the treatment groups; (4) Identification and description of adverse events; (5) The treatment group received orally administered HM; (5) The control groups received placebo, CM or no treatment.

We excluded studies in which HM was used in combination with CMs, in children and in control groups. Administration of HM by other routes such as injections were also excluded.

Information on HM products derived from a single herb, Chinese proprietary medicines, complex extracts of different herb preparations such as decoction, tablet, capsule, pill, powder and plaster were collected. Standardized extracts of whole plants were included, but isolated 'active' phytochemical ingredients were excluded as these are generally considered as plant chemical products.

Data extraction

Data was collected independently by the reviewers. Any disagreement between the reviewers was resolved by discussion in order to achieve consensus.

Assessment of study quality

The reviewers also assessed independently the quality of each study by Jadad scale[8] and Cochrane Handbook[9].

Statistical analysis

The relative risk (RR) and 95% confidence interval (95% CI) were calculated using raw data derived from each study. Intention-to-treat analysis was performed if possible. Meta-analyses was performed with either fixed effects model or random effects model according to the presence or absence of heterogeneity when HM was compared with control. Statistical analysis was performed with RevMan 4.2 to detect any bias in studies using the funnel plot.

RESULTS

Our initial search generated 572 citations. After analyzing the titles and abstracts, and reading the full text articles, only 22 studies[10-31] with 25 arms involving 1279 patients and 763 controls met the predefined inclusion criteria. Eighteen of the studies were conducted in China and published in Chinese, four were published in English, one each from Australia[11] and Israel[20], and two from Germany[29,31] (Table 1).

Three studies[11,20,29] used computer software and two[21,25] used random number tables to generate the allocation sequence. Four studies[11,20,29,31] used an adequate concealed allocation method of randomization. Six studies[11-12,20,27,29,31] used placebo as control, four of these studies[11,20,29,31] were considered to be adequate, while two[12,27] demonstrated an inadequate comparison between placebo and CTM decoction; Four studies[11,20,29,31] provided statistical data with intention-to-treat protocol.

Using the Jadad Score and Cochrane handbook, four studies[11,20,29,31] were judged to be of high quality, whereas the remaining reports were of poor quality.

Efficacy of herbal medicines

Global symptoms of IBS: Two studies[11,29] with 3 HMs showed significant benefit compared to placebo with respect to the global improvement of IBS symptoms: standard CHM formula[11] (RR 2.15; 95% CI: 1.26-3.65 rated by patient and RR 2.62; 95% CI: 1.44-4.78 assessed by gastroenterologist), STW5[29] (RR 1.68; 95% CI: 1.13-2.51), STW5-Ⅱ[29] (RR 1.90; 95% CI: 1.30-2.78) (Figure 1). The following seven CHMs using complex herbal formulas appeared to be effective in improving global IBS symptoms compared to CM: Lizhong huoxie decoction[10] (RR 1.40; 95% CI: 1.11-1.76), Huatan Liqi Tiaofu decoction[13] (RR 1.24; 95% CI: 1.05-1.47), Geqin Shujiang Saocao decoction[14] (RR 1.24; 95% CI: 1.05-1.47), Huanchang decoction[16] (RR 1.41; 95% CI: 1.08-1.84), Congpi Lunzhi Formula[17], (RR 1.74; 95% CI: 1.35-2.24), Xiangsha Liujunzi decoction[18] (RR 1.28; 95% CI: 1.00-1.63, $P = 0.015$), and Shunji mixture[19] (RR 1.23; 95% CI: 1.01-1.49).

By contrast, the following compounds were not effective in the treatment of global IBS symptoms compared to placebo or CM: Individualized CHM[11] (RR 1.51; 95% CI: 0.83-2.73 assessed by patients and RR 1.54; 95% CI: 0.77-3.05 assessed by gastroenterologist), Bitter candytuft[29] (RR 1.23; 95% CI: 0.78-1.92), Curcuma [31] (RR 0.97; 95% CI: 0.60-1.55), Fumitory[31] (RR 1.13; 95% CI: 0.74-1.72) (Figure 1), Changkang Capsule[21] (RR 1.03; 95% CI: 0.92-1.14), and Jiejing Yiji decoction[30] (RR 1.09; 95% CI: 0.96-1.23) (Figure 2).

Diarrhea: As shown in Figure 3, a meta-analysis of Tongxie Yaofang modified decoction[12] and Tongxie Yaofang plus Sini San decoction[27] (RR 1.14; 95% CI: 1.04-1.24) showed that compared to CM these products had antidiarrheal effects in patients with diarrhea-predominant IBS patients. We combined the data from these two studies[12,27] since their herbal ingredients and dosages were very similar. Their effects were similar to Liyiting decoction[26] (RR 1.28; 95% CI: 1.02-1.62), and Tongxie yihao capsule[28] (RR 1.22; 95% CI: 1.01-1.46).

Three studies demonstrated an insignificant improvement in diarrhea: Xianshi Capsule[15] (RR 1.20; 95% CI: 0.98-1.48), Changning Yin decoction[24] (RR 1.19; 95%

Table 1 Characteristics of studies included in the analysis

Study ID	Year	*n*	Mean age	Sex (male %)	Study quality	Herbal medicine	Type of herbs	Control	Length (wk)	Follow-up (wk)
Gao[10]	1992	111	34	39	L	Lizhong Huoxie decoction	C.F	Oryzanol, Nifedipine	4	52
Bensoussan[11]	1998	116	47	35	H	Individualized CHM Standard Formula	C.F C.F	Placebo	16	14
Zhao[12]	2000	233	39	34	L	Tongxie Yaofang modified decoction	C.F	Salazosulfapyridine Diphenoxylate, Anisodamine, Amitriptyline, Placebo	3	n.r
Lei[13]	2000	96	39	56	L	Huatan Liqi Tiaofu decoction	C.F	Smecta	3	n.r
Wang[14]	2000	96	39	49	L	Geqin Shujiang Shaocao decoction	C.F	Smecta, vitB	3	n.r
Ye[15]	2002	80	37	46	L	Xianshi Capsule	C.F	Dicetel, Smecta	8	n.r
Deng[16]	2002	62	38	35	L	Huanchang decoction	C.F	Anisodamine, Oryzanol	3-6	52
Zeng[17]	2002	98	38	46	L	Congpi Lunzhi Formula	C.F	Bacillus Licheniformis	6	n.r
Ge[18]	2002	57	40	44	L	Xiangsha Liujunzi decoction	C.F	Diazepam, Propantheline, Domperidone	2	n.r
Zhou[19]	2002	105	n.r	n.r	L	Shunji mixture	C.F	Bitinal	4	n.r
Sallon[20]	2002	80	47.9 ± 2.1[1] 46.3 ± 2.9[3]	38	H	Padma Lax (Tibetan herbal formula)	C.E	Placebo	12	n.r
Ma[21]	2003	204	36	48	L	Changkang Capsule	C.F	Amitriptyline	4	n.r
Wang[22]	2003	104	53	53	L	Wuma Simo decoction	C.F	Cisapride	2	n.r
Liu[23]	2003	77	37	36	L	Gegan Qinlian Pellet	C.F	Nifedipine	3	n.r
Li[24]	2003	101	39	68	L	Changning Yin decoction	C.F	Diphenoxylate	5	n.r
Shen[25]	2003	47	41.6 ± 12.8[1] 42.3 ± 14.7[3]	57	L	Changjitai decoction	C.F	Dicetel	8	n.r
Zhao[26]	2004	84	44	44	L	Liyiting decoction	C.F	Dicetel	6	n.r
Xiao[27]	2004	167	37	41	L	Tongxie Yaofang plus sini san decoction (similar with Tongxie Yaofang modified decoction)	C.F	Salazosulfapyridine Diphenoxylate, Anisodamine, Amitriptyline, Placebo	2	n.r
Gao[28]	2004	98	36	35	L	Tongxie yihao capsule	C.F	Dicetel, Domperidone, Loperamide, Doxepin	4	24
Madisch[29]	2004	208	47	40	H	1 STW5 2 STW5- Ⅱ 3 Bitter Candytuft	C.E C.E M.E	Placebo	4	n.r
Bo[30]	2004	92	38.8 ± 1.7[1] 41.3 ± 1.7[3]	41	L	Jiejing Yiji decoction	C.F	Cerekinon	2	4
Brinkhaus[31]	2005	106	47.2 ± 11.7[1] 49.5 ± 14.5[2] 49.0 ± 9.1[3]	37	H	1 Curcama 2 Fumitory	M.E M.E	Placebo	18	n.r

H: High quality study; L: Low quality study; C.F: Complex formulation of herbs; C.E: Complex extracts of different herbs; M.E: Mono-extract of single herb. [1]The first treatment group; [2]The second treatment group; [3]The control group; n.r: Not reported.

CI: 0.99-1.42), Changjitai decoction[25] (RR 1.14; 95% CI: 0.81-1.60) (Figure 3).

Constipation: One study[20] showed that compared to placebo Padma Lax was more effective in relieving symptoms in patients with constipation-predominant IBS (RR 7.24; 95% CI: 2.37-22.12) (Figure 4). Similarly, another study[23] demonstrated that Gegan Qinlian pellet was therapeutically effective (RR 1.28; 95% CI: 1.03-1.60). By contrast, Wuma Simo decoction[22] had no advantages over CMs in the patients with constipation-predominant IBS (RR 1.07; 95% CI: 0.91-1.25) (Figure 5).

Therapeutic period and follow-up

The duration of treatment was 8 weeks or more in five studies[11,15,20,25,31], and 5 or 6 wk in three studies[17,24,26]. In one study[16] the treatment duration was 3-6 wk, whereas the other studies[10,12-14,19,21,23,28,29] lasted 3 or 4 wk, while the shortest[18,22,27,30] period of the treatment was only 2 wk. Five studies[10-11,16,28,30] reported follow-up assessment after herbal medicine treatment. Two[10,28] reported that the symptom recurrence rates were lower in the treatment group compared to the conventional treatment group (25.5% *vs* 60%; 23.1% *vs* 50%, $P < 0.01$), after one-year and one-half-year respectively, following completion of the treatment. Another study[11] presented the result as bowel symptom scale. There was significant improvement in the individualized group (75%), and standard group (63%) compared with placebo group (32%) after 14 wk of follow-up. One study[16] reported the number of subjects that were lost to follow-up, but the reasons were not provided. Another study[30] reported symptom recurrence in 3 of 48 patients in the treatment group after 2 wk without treatment.

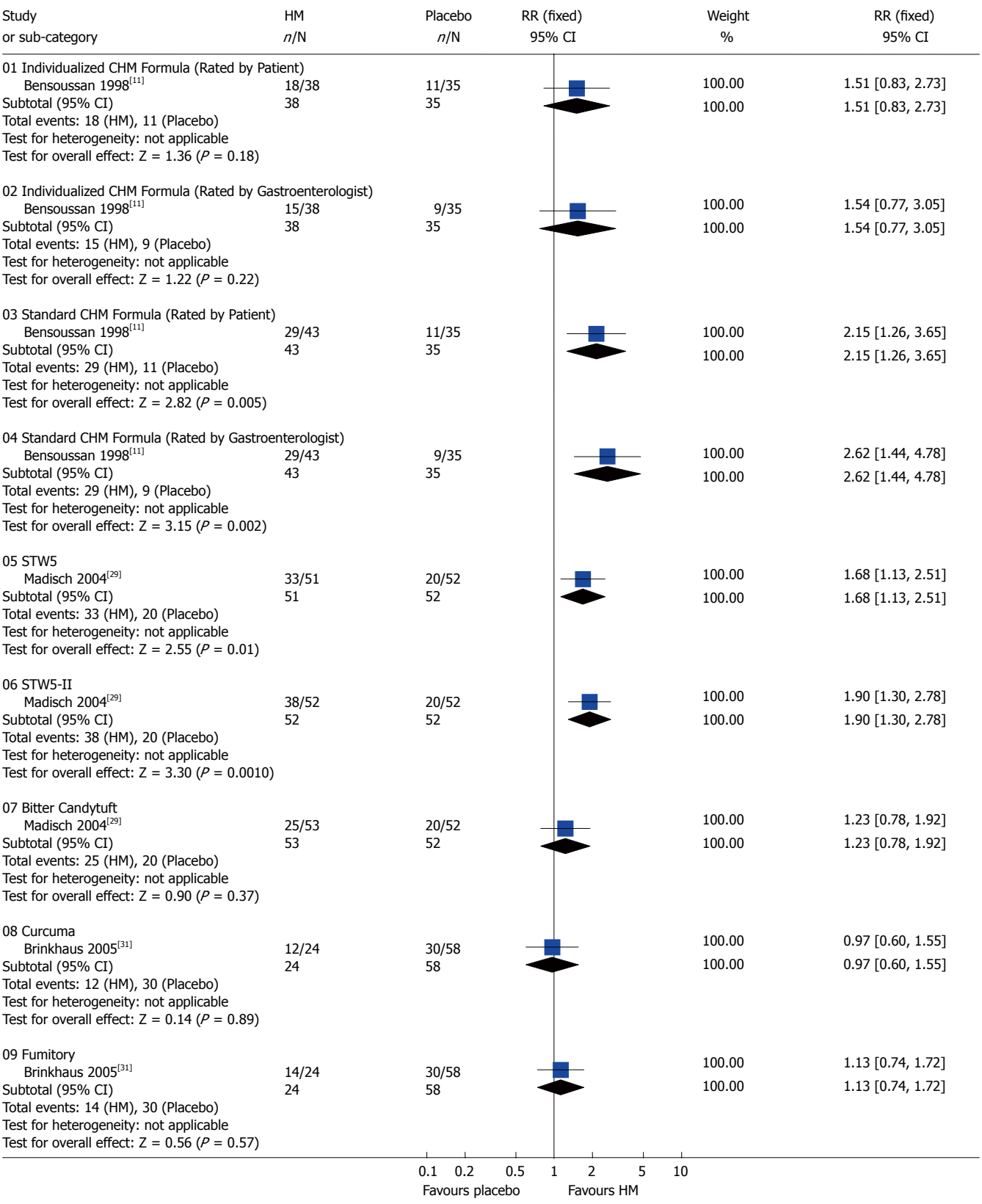

Figure 1 Comparison of herbal medicine and placebo (Outcome: global improvement of symptoms). HM: Herbal medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

Adverse effects of herbal medicine

In the total study group of 1279 patients, only 15 adverse events in 47 subjects were observed (Table 2). The most common symptoms were abdominal distention, constipation and abdominal pain. None of the subjects developed any serious adverse events or abnormal laboratory tests. The percentage of adverse events associated with HM was 3.67% (95% CI: 2.64-4.71%) in the 1279 patients in the different treatment groups.

Bias analysis

Funnel plots indicated an asymmetry (Figure 6).

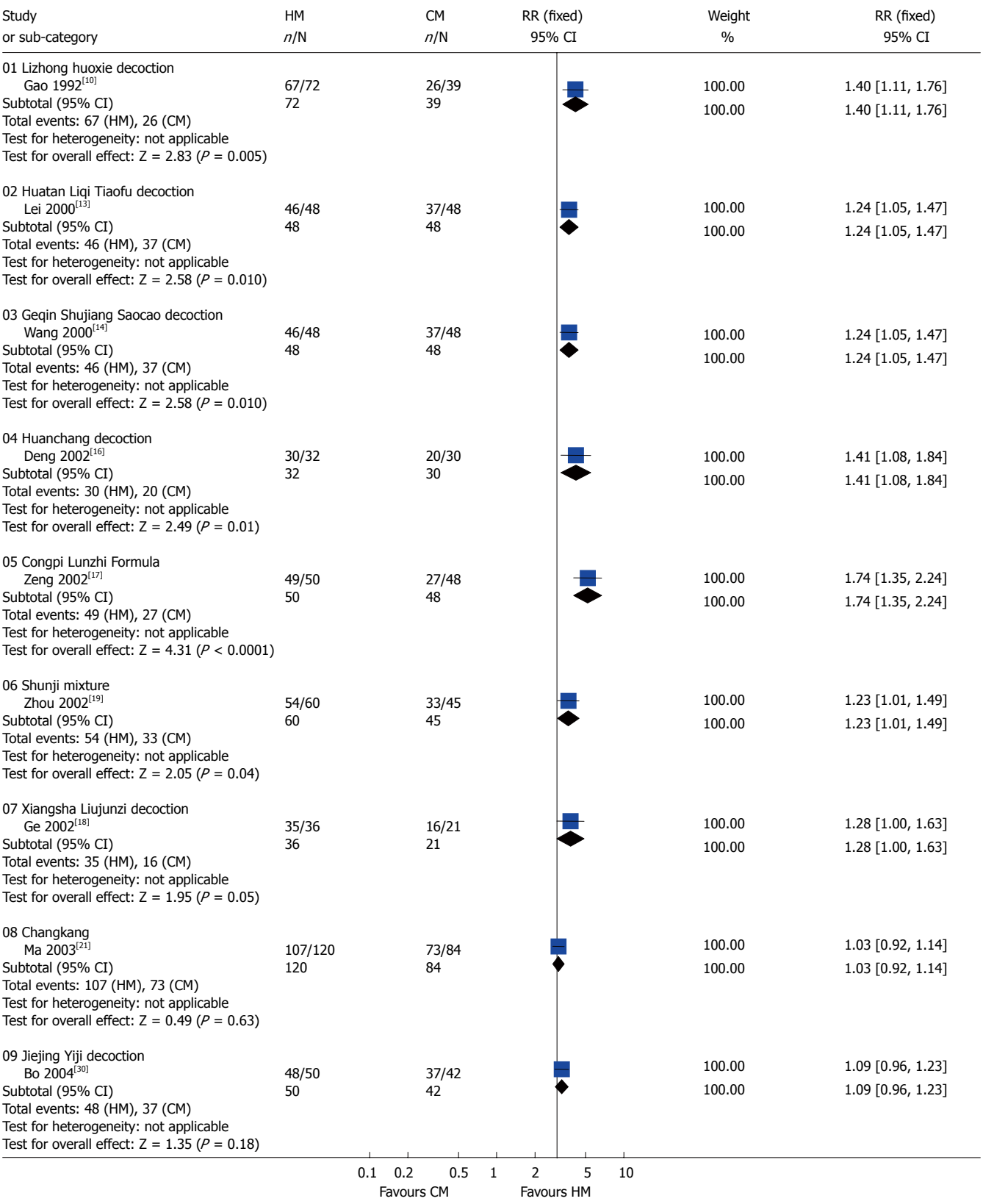

Figure 2 Comparison of herbal medicine and conventional medicine (Outcome: Global improvement of symptoms). HM: Herbal medicine; CM: Conventional medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

DISCUSSION

In the present review, 3 out of the 4 good quality studies[11,20,31] demonstrated 4 different herbal interventions: one Chinese herbal medicine (standard formula), one Tibetan herbal formula (Padma Lax) and two complex extracts of herbs: STW5 and STW5-Ⅱ which could potentially relieve abdominal pain, constipation, diarrhea and alternating constipation and diarrhea. Moreover, three[11,29,31] out of these four studies showed that four

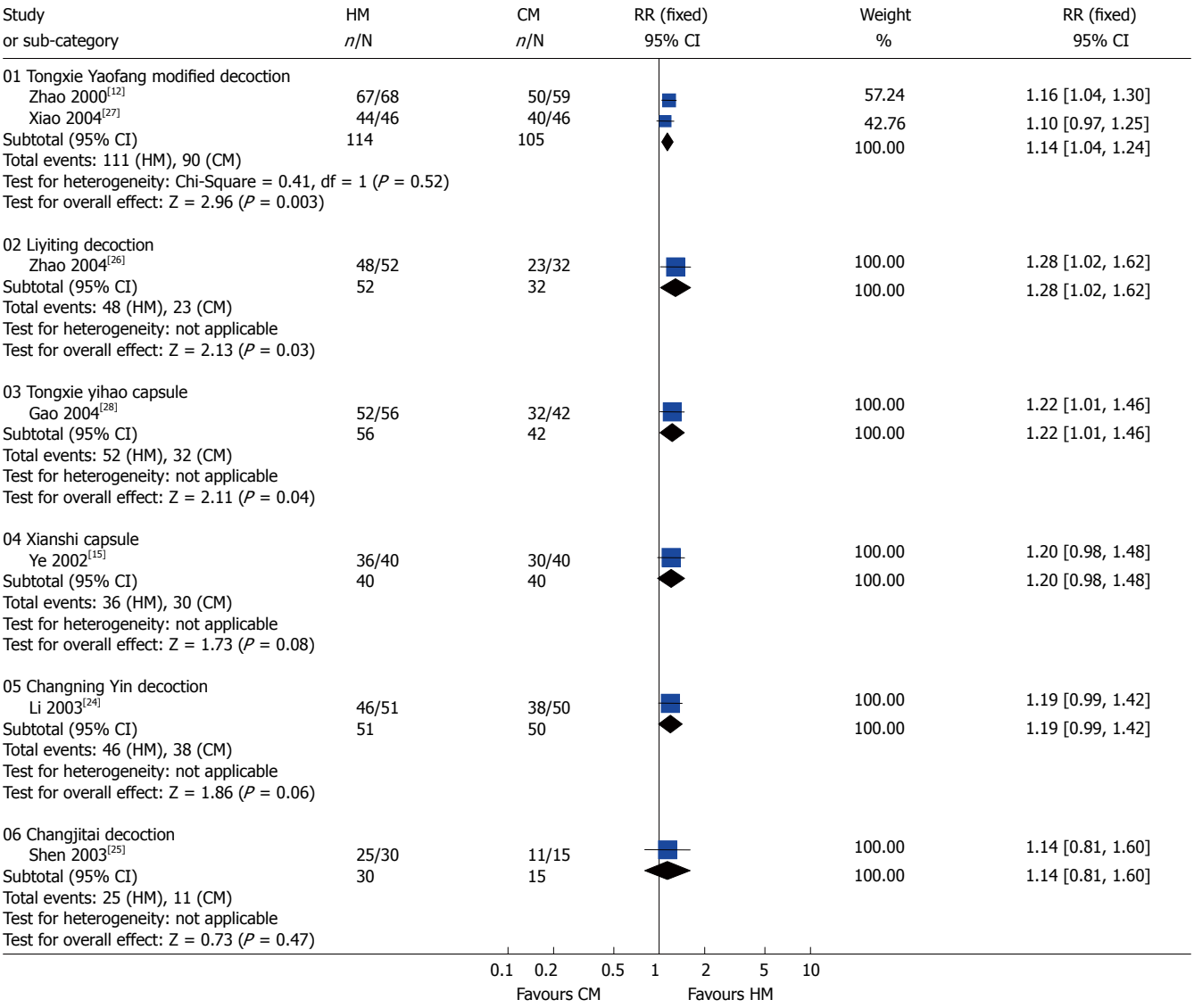

Figure 3 Comparison of herbal medicine and conventional medicine (Outcome: diarrhea). HM: Herbal medicine; CM: Conventional medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

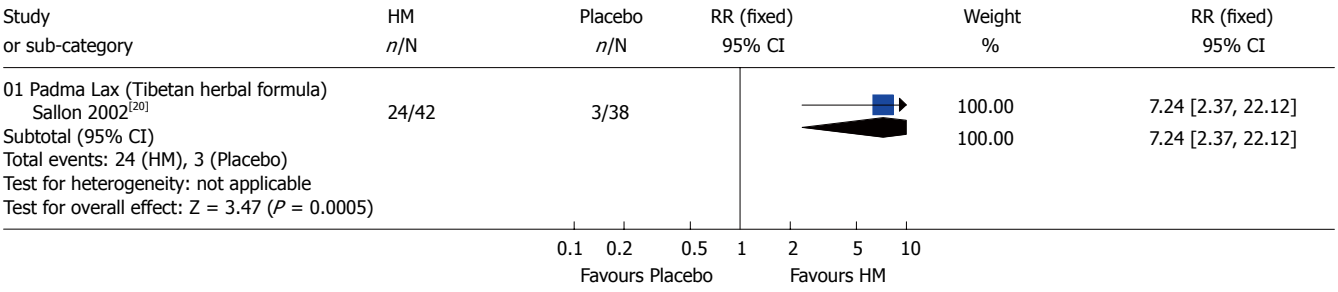

Figure 4 Comparison of herbal medicine and placebo (Outcome: constipation). HM: Herbal medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

interventions including one individualized formula and three mono-extracts of single herb (Bitter Candytuft, Curcama and Fumitory) were not effective in IBS. We recognized that some complex herbal formulas may improve IBS symptoms, whereas three mono-extracts of single herbs had no beneficial effect. A possible explanation for these findings is that the therapeutic effect may be enhanced by the synergic actions of compounds in a mixture of different herbs.

Twelve[10,12-14,16-19,23,26-28] out of the 18 poor quality studies showed that some Chinese herbs formulas, such as, Huatan Liqi Tiaofu decoction, Tongxie Yaofang modified and Tongxie Yaofang plus Sini San decoction, Geqin Shujiang Saocao decoction, Huanchang decoction, Congpi Lunzhi Formula, Xiangsha Liujunzi decoction, Shunji mixture, Gegan Qinlian Pellet, and Liyiting decoction were more beneficial than CMs in the treatment of IBS.

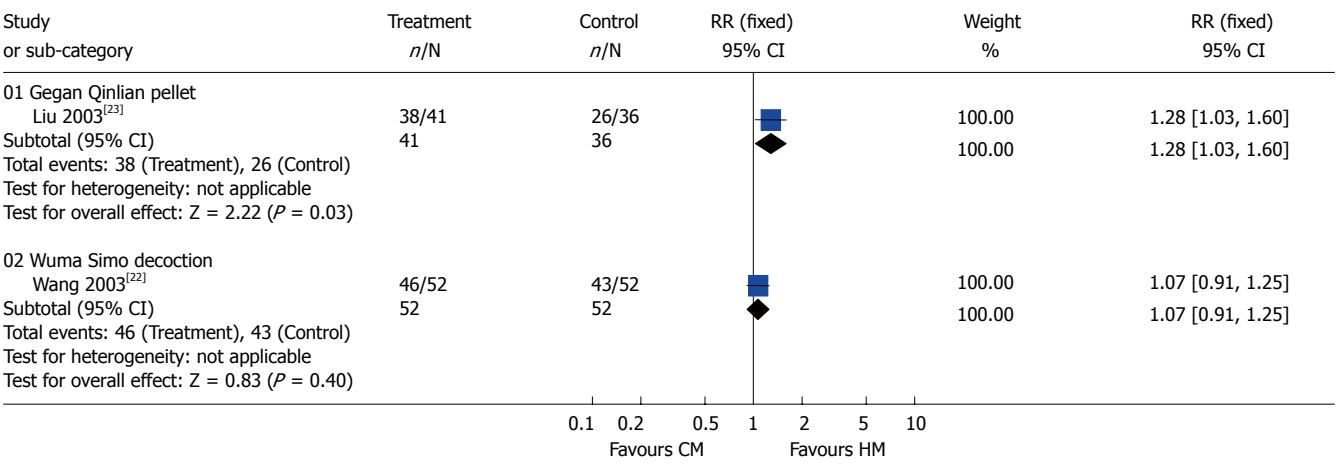

Figure 5 Comparison of herbal medicine and conventional medicine (Outcome: constipation). HM: Herbal medicine; CM: Conventional medicine; RR: Relative risk; CI: Confidence interval; Fixed: Fixed effects model.

Table 2 The type and frequency of adverse events reported in the 22 studies included in the analysis (n = 1279)

Adverse events	Number of adverse events	Percentage	95% CI
Distention	9	0.70	0.32-1.34
Diarrhea	8	0.63	0.27-1.23
Abdominal pain	6	0.47	0.17-1.02
Constipation	5	0.39	0.13-0.91
Dizziness and sleepiness	4	0.31	0.09-0.80
Headaches	4	0.31	0.09-0.80
Nausea	3	0.23	0.05-0.69
Gastrointestinal discomfort	1	0.08	0.002-0.44
Upper gastrointestinal discomfort	1	0.08	0.002-0.44
Loss of hair	1	0.08	0.002-0.44
Pruritus	1	0.08	0.002-0.44
Paraesthesia	1	0.08	0.002-0.44
Disturbance	1	0.08	0.002-0.44
Hoarseness	1	0.08	0.002-0.44
Shortness of breath and chest pain	1	0.08	0.002-0.44
Total	47	3.67	2.73-4.87

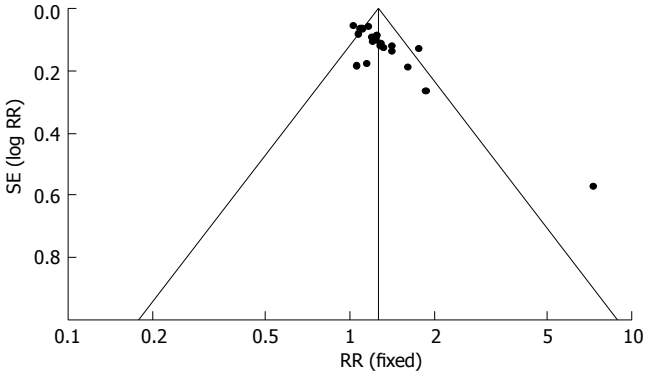

Figure 6 Funnel plot. Fixed: Fixed effects model; RR: Relative risk.

However, these studies revealed several methodological flaws. We found that the longest duration of treatment was 18 wk, while the shortest was just 2 wk, and only 5 studies lasted more than 8 wk. The most frequent treatment duration was 3-4 wk. With such a short period of treatment, it is hard to reach the therapeutic goal in IBS. Some studies reported the long-term effects of herbs and the rate of symptom recurrence. Thus, it is necessary to have a relatively long treatment duration with herbal medicines as well as the duration of the follow-up period; Funnel plot of inclusion trials indicated asymmetry, the major interpretation is the presence of publication bias and variable methodological quality. In the studies that we reviewed, 18 out of 22 were conducted in China and published in Chinese. Chinese studies more frequently showed favorable therapeutic results compared to articles in English, particularly those with a high rate of positive outcome (99%)[32]. The large number of poor quality studies is another source of bias. Furthermore, the small size of studies and the variability of the control treatment may cause asymmetry of the funnel plot. We noticed that most of the studies with Chinese herbs were of poor methodological quality and would not provide strong evidence to confirm the efficacy of CHM. However, the lack of good evidence supporting the effectiveness of CHM does not mean that these preparations are not effective in the treatment of IBS, instead we need to improve the methodological quality of trials in order to verify the efficacy of CHM as a therapeutic approach. We agree with the opinion of Liu[33] that the potential beneficial effects of CHM need to be confirmed in rigorous trials with well-designed, randomized, double blinded, placebo controlled studies. A good example is the study performed by Bensoussan and colleague[11].

There is growing interest in placebo response in patients with IBS. A systematic review of RCTs showed that global improvement in IBS symptoms with placebo was 40.2% (range 16%-71.4%)[34].Other investigators have reported placebo response rate of 57% in IBS[35]. Placebo response rate correlated with factors such as frequency of intervention, methodological quality of study, duration of the study, the patient-practitioner interaction and the diagnosis treated[34,36-38]. Since the number of studies using placebo were small, we did not explore the response effect of placebo in the present study.

In the 22 trials that we reviewed, there were only 15 adverse events associated with HM. These were abdominal distention, constipation, abdominal pain, diarrhea, dizziness and hypersomnia, headache and nausea. However, no serious side effects or abnormalities of laboratory parameters such as liver function, renal function or haematological tests were reported with the treatment. Studies conducted in the West reported more adverse events than those from China. It is possible that because of the lack of rigorous monitoring, several adverse effects including serious events may not have been reported. Similarly, because of publication bias, adverse events related to herbal medicine may not be reported properly.

In summary, the use of HM for treating IBS is increasing worldwide. Most of the studies included in our review showed a beneficial effect on IBS symptoms. However, the methodological quality of the studies was variable, with 82% being of poor quality which may have overestimated the effectiveness of treatment. Although adverse events arising from the use of herbs were mild and infrequent, HM should be used with caution because of the reasons discussed above. It is therefore necessary to conduct Level I studies in order to provide evidence for Grade A recommendations[39] and clarify whether Chinese herbal medicines are reliable and safe therapy in IBS.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional bowel disorder that affects the patient's quality of life. No single treatment is reliably effective, and an increasing number of patients worldwide are seeking herbal medicines to cure their illness.

Research frontiers

We assessed the efficacy and adverse events of herbal medicines in IBS.

Innovations and breakthroughs

We made a comprehensive search of studies using herbal medicines for the treatment of IBS. The studies were analyzed to determine if herbal medicines are appropriate for IBS patients.

Applications

Based on this evaluation, we concluded that herbal medicines could not be reliably recommended because of methodological flaws in the studies. Further studies of better methodological quality should be carried out to determine the efficacy of herbal medicines in IBS.

Peer review

The authors explored the efficacy and safety of herbal medicines (HM) in the treatment of IBS. It was concluded that herbal medicines have therapeutic benefit in IBS.

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RAPID COMMUNICATION

Enteral glutamine pretreatment does not decrease plasma endotoxin level induced by ischemia-reperfusion injury in rats

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Abstract

AIM: To investigate whether oral glutamine pretreatment prevents impairment of intestinal mucosal integrity during ischemia-reperfusion (I/R) in rats.

METHODS: The study was performed as two series with 40 rats in each. Each series of animals was divided into four groups. The first group was used as a control. Animals in the second group were only pretreated with oral glutamine, 1 g/kg for 4 d. The third group received a normal diet, and underwent intestinal I/R, while the fourth group was pretreated with oral glutamine in the same way, and underwent intestinal I/R. Intestinal mucosal permeability to ^{51}Cr-labeled EDTA was measured in urine in the first series of animals. In the second series, histopathological changes in intestinal tissue and plasma endotoxin levels were evaluated.

RESULTS: Intestinal I/R produced a significant increase in intestinal permeability, plasma endotoxin level and worsened histopathological alterations. After intestinal I/R, permeability was significantly lower in glutamine-treated rats compared to those which received a normal diet. However, no significant change was observed in plasma endotoxin levels or histopathological findings.

CONCLUSION: Although glutamine pretreatment seems to be protective of intestinal integrity, upon I/R injury, such an effect was not observable in the histopathological changes or plasma endotoxin level.

Key words: Endotoxin; Glutamine; Intestinal permeability; Ischemia-reperfusion injury

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INTRODUCTION

Intestinal injury as a result of ischemia and subsequent reperfusion plays an important role in a variety of clinical conditions such as shock, and in those undergoing cardiovascular surgery[1]. In these situations, the small intestine may suffer ischemia of varying duration. Intestinal ischemia-reperfusion (I/R) disrupts intestinal mucosal integrity[2,3], and causes an increase in intestinal permeability[4,5] and bacterial translocation[6]. Plasma endotoxin level also increases after I/R[6]. All these have been increasingly recognized as potential causes of multi-system organ failure in critically ill patients[7-9].

Intestinal permeability is assessed non-invasively *in vivo* by measuring urinary excretion of orally administered test substances. Lactulose, various polymers of polyethylene glycol, ^{51}Cr-labeled EDTA and ^{99m}Tc diethylenetriaminepentaacetate are the most commonly used test substances. Test results may be influenced by a change in any of the pre- or post-mucosal factors, apart from intestinal permeability itself[10]. Measurement of intestinal permeability by some test substances may give an idea about some harmful factors such as microorganisms passing through the mucosal barrier, from the intestinal lumen to the systemic circulation. It is thought an increase in plasma endotoxin level is one of the responsible factors which may contribute to the clinical results of intestinal I/R injury[11]. Measurement of plasma endotoxin levels seems to be important in assessing the systemic effect of intestinal I/R.

Glutamine (Gln) is a non-essential amino acid, and the most abundant free amino acid in whole blood and the

intracellular amino acid pool[12]. It is an important respiratory fuel, and nucleotide precursor for the gastrointestinal tract[13]. Gln-supplemented parenteral nutrition protects rats against morphological and functional mucosal injury[14], and improves survival in animals after intestinal I/R[15]. Some other experimental studies have also shown that intraluminal injection of Gln protects the mucosa, and diminishes the accumulation of neutrophils in the lamina propria of the small bowel during I/R[16]. Generation of reactive oxygen intermediates (ROI) during reperfusion is thought to be one of the major causes of intestinal mucosal injury[17,18]. Gln is also essential for the synthesis of the intrinsic ROI scavenger glutathione. Therefore, this protective action may be due to augmentation of ROI scavenging in intestinal mucosa[19].

In this study, we aimed to investigate the effects of orogastric Gln pretreatment on intestinal mucosal permeability, plasma endotoxin level and intestinal histopathology during intestinal I/R injury in rats.

MATERIALS AND METHODS

Experimental design

The experiment was performed in female Wistar albino rats weighing 200-230 g. It was approved by the Ankara University Ethical Committee, and was conducted according to the European Community guidelines for the use of experimental animals. Animals were fed with their ordinary diet and allowed to drink water *ad libitum*. Owing to the time difference between urine collection and blood sampling, the study had to be completed in two series of experiments (Table 1).

Table 1 Characteristics of study groups

Groups	Measurement of intestinal permeability by ^{51}Cr-EDTA		Measurement of plasma endotoxin levels and histopathological changes	
	Gln	I/R	Gln	I/R
Ⅰ (Control)	–	–	–	–
Ⅱ (Gln)	+	–	+	–
Ⅲ (I/R)	–	+	–	+
Ⅳ (I/R and Gln)	+	+	+	+

Time-matched, sham-operated animals undergoing laparotomy and dissection of the SMA without occlusion served as controls (group Ⅰ). The Gln group (group Ⅱ) was pretreated with Gln (1 g/kg per day) by the orogastric route for 4 d[20,21]. Gln was prepared in 0.9% NaCl for daily use. Intestinal I/R group (group Ⅲ) underwent 1 h intestinal ischemia, and 1 h reperfusion. In the I/R and Gln group (group Ⅳ), I/R periods and Gln administration were the same as in Gln (Ⅱ) and I/R (Ⅲ) groups.

Induction of intestinal I/R

A rat model of transient mesenteric occlusion was used to obtain intestinal I/R. Rats were anesthetized with ketamine (80 mg/kg, im) and xylazine (10 mg/kg, im). Intestinal I/R was induced by 60 min occlusion, followed by 60 min reperfusion[22]. During the 2 h of the surgical procedure, animals were kept at room temperature, and given intraperitoneal fluid as 0.9% NaCl (10 mL/kg). The superior mesenteric artery (SMA) was exposed through a midline abdominal incision, and both this artery and the collateral branches coming from the celiac axis, and the inferior mesenteric artery were occluded with atraumatic vascular clamps (Vascu-Statt Ⅱ original No. 1001-532-3; Scanlan International, St Paul, MN, USA) for 1 h, followed by 1 h reperfusion. Existence of pallor and absence of pulsation ensured mesenteric occlusion during the ischemic period. Recovery of pulsation and pink color were controlled in each animal when the clamps were removed. The existence of intestinal I/R in this model was also confirmed in our laboratory by the appearance of pulses at the marginal arteries (direct vision of mesenteric circulation by microscopy), as well as by fluorescein angiography in preliminary experiments[23].

Measurement of intestinal mucosal permeability

Intestinal mucosal permeability was measured on the basis of urinary radioactivity levels following oral administration of ^{51}Cr-EDTA. ^{51}Cr-EDTA was employed as a well accepted marker of mucosal integrity[24,25]. After 60 min reperfusion, rats were given 5 μCi ^{51}Cr EDTA in 0.5 mL saline solution by the orogastric route. Urine samples were collected in metabolic cages for 6 h following the reperfusion period. During urine collection, animals did not receive any food; however, they were allowed to access tap water. The level of radioactivity in the urine samples of 500 μL was then determined by counting on a gamma counter (DPC Gambyt CR, Los Angeles. USA). The amount of ^{51}Cr-EDTA excreted in urine during 6 h was calculated as a percentage of the ingested dose.

Measurement of plasma endotoxin level

Plasma endotoxin level was measured by the colorimetric Limulus amebocyte lysate (LAL) test. The test was performed by using the Pyrochrome test kit (Pyroquant Diagnostik, Mörfelden, Germany). All glassware, solutions and surgical instruments used in the experiment were autoclaved at 121℃ for 15 min. The non-pyrogenicity of solutions was tested using the LAL test (Charles River Endosafe, Charleston, SC, USA).

Venous blood samples (3-4 mL) were collected using heparin-coated pyrogen-free disposable syringes. Platelet-rich plasma (PRP) was prepared from the blood by centrifugation at 150 g for 10 min. Fifty microliters of PRP was transferred into a polystyrene plastic tube and kept frozen at -80℃ until the assay. Frozen PRP samples were kept at room temperature for about 30 min before the assay. Fifty microliters of 0.18 mol/L NaOH was added to 50 μL PRP, and incubated at 37℃ for 5 min. Next, 50 μL 0.32 mol/L perchloric acid was added, and incubated at 37℃ for a further 10 min. To dissolve the formed precipitate, 100 μL 0.18 mol/L NaOH was added, and vortexed. Twenty-five microliters of the solution was transferred into sterile non-pyrogenic microplates (Pyroquant Diagnostik), and 25 μL 0.2 mol/L Tris/HCl buffer (pH 8.0) was added to the wells[26]. Finally, 50 μL pyrochrome test solution was added to all wells, and mixed for 30 s. Plates were incubated at 37℃. Optical density was read at 405 nm. Standard curves from 0.04 to 1.28 EU/mL were used to evaluate the concentration of endotoxin.

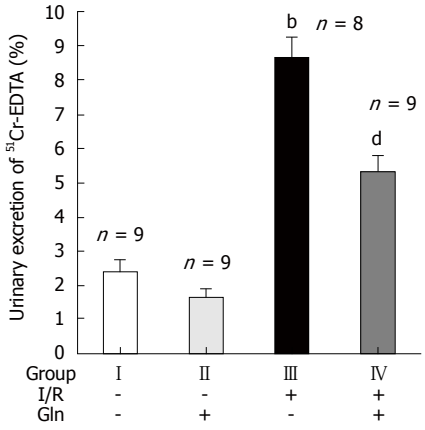

Figure 1 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on intestinal permeability during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Changes in intestinal permeability were measured by urinary excretion of ^{51}Cr-EDTA after its oral administration in rats. Data are expressed as means ± SEM. $^{b}P < 0.001$ *vs* group Ⅰ, Ⅱ and Ⅳ; $^{d}P < 0.001$, *vs* groups Ⅰ, Ⅱ and Ⅲ.

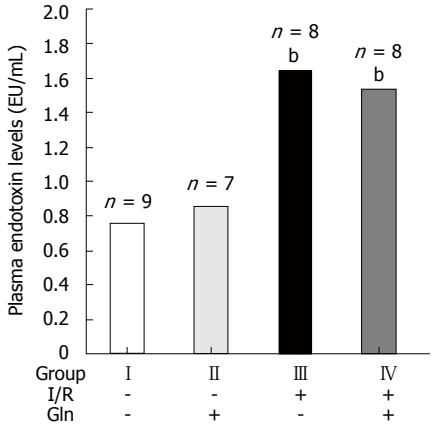

Figure 2 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on plasma endotoxin levels during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Changes in plasma endotoxin levels were measured by LAL test. Data are expressed as medians. $^{b}P < 0.001$ *vs* groups Ⅰ and Ⅱ.

Results were calculated by using non-linear regression of a four-parameter logistic model.

Histopathological assessment of ileal tissues

After the collection of blood samples, ileal tissue samples, 10 cm proximal to the cecum, were harvested and evaluated for histopathological changes. Sections were stained with hematoxylin and eosin, and were examined by light microscopy by two pathologists in a blinded manner. Mucosal injury was scored on a scale from 0 to 5, as described by Chiu *et al*[2].

Statistical analysis

The SPSS program was used for statistical analysis. Comparison of the various protocols on the changes in intestinal permeability was made by one-way analysis of variance (ANOVA) following a Bonferroni post-hoc test. Changes in plasma endotoxin levels, and intestinal histopathology were determined using a Kruskal-Wallis test following a multiple comparison post-hoc test[27]. Data on changes in intestinal permeability were presented as means ± SEM. Data of plasma endotoxin levels, and intestinal histopathology were presented as medians. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Gln pretreatment on intestinal permeability during I/R injury

To investigate the effect of intestinal I/R and Gln pretreatment on intestinal permeability, renal clearance of ^{51}Cr-EDTA was assessed. Statistically significant differences were detected in intestinal permeability in the I/R group when compared to the control group (8.6% ± 1.7% *vs* 2.4% ± 1.1%, $P < 0.001$). Gln pretreatment significantly lowered the increased intestinal permeability due to intestinal I/R (8.6% ± 1.7% *vs* 5.3% ± 1.3%, $P < 0.001$). There was no statistically significant difference in intestinal permeability between the control and Gln groups (Figure 1).

Effect of Gln pretreatment on plasma endotoxin level during I/R injury

In the second series of experiments, plasma endotoxin level was evaluated after intestinal I/R injury. There was a statistically significant difference in plasma endotoxin level between the control (group Ⅰ), and intestinal I/R injury (group Ⅲ) groups (0.76 EU/mL *vs* 1.64 EU/mL, $P < 0.001$). Enteral Gln pretreatment lowered the plasma endotoxin level (group Ⅳ), which was increased due to intestinal I/R injury (group Ⅲ) (1.54 EU/mL *vs* 1.64 EU/mL, $P = 0.48$). However, this decrease failed to reach statistical significance. There was no significant difference in plasma endotoxin levels between the control (group Ⅰ), and Gln (group Ⅱ) groups (0.76 EU/mL *vs* 0.86 EU/mL, $P = 0.59$) (Figure 2).

Effect of Gln pretreatment on intestinal histopathological changes during I/R injury

Intestinal histopathological changes were also evaluated in the second series of experiments. There was significant intestinal injury due to intestinal I/R when the control group was compared to the I/R group (0 *vs* 3, $P < 0.001$). Gln pretreatment did not significantly decrease intestinal injury compared to the I/R group (3 *vs* 2, $P = 0.389$). There was no significant difference in histopathological changes between the control and Gln groups (Figure 3).

DISCUSSION

Ischemia and subsequent reperfusion is one of the major causes of cell injury. The mechanisms of I/R injury are complex, and are likely to differ with respect to the duration of ischemia, the specific tissue involved, and the species studied[3,28]. The small intestine may experience ischemia and reperfusion during septic shock[29], hemorrhagic shock[30] or cardio-vascular surgery[31,32]. These clinical situations can result in some serious postoperative complications such as delay in anastomotic healing[33]. Intestinal mucosa is known to be sensitive to I/R injury. Its basal high rate of oxygen use renders the intestine relatively incapable of increasing oxygen transport in cases of hypoxic stress, and thus is

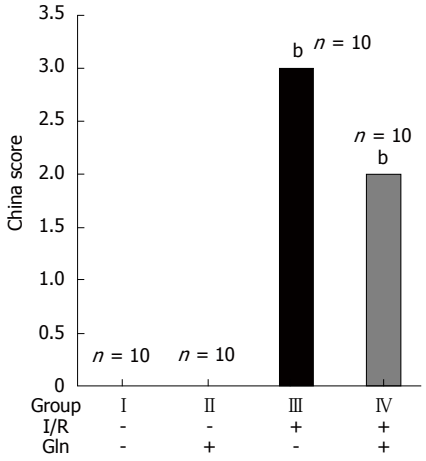

Figure 3 The effect of enteral Gln pretreatment (1 g/kg per day for four days) on histopathological changes during I/R injury in rats. Intestinal I/R was induced by 60 min of occlusion, followed by 60 min of reperfusion. Histopathological changes were scored by the "Chiu" intestinal ischemia scoring system. Data are expressed as medians. $^{b}P < 0.001$ *vs* groups Ⅰ and Ⅱ.

more susceptible to ischemic injury. Intestinal ischemia predisposes the gut to subsequent necrosis. Restoration of blood flow and reintroduction of oxygen after deprivation accelerates tissue injury[34]. Reperfusion of ischemic intestine may lead to more severe functional and morphological changes than the injury produced by ischemia itself[3]. Oxygen free radicals are thought to be generated during the reperfusion phase, with the addition of oxygen to previously ischemic tissues causing the formation of reactive oxygen species[35].

Intestinal I/R disrupts the functional intestinal mucosal barrier, which results in an increase in mucosal permeability to ^{51}Cr-EDTA[5]. The results of the present study also demonstrated a significant increase in intestinal permeability to ^{51}Cr-EDTA, due to intestinal I/R injury (Figure 1). An increase in mucosal permeability also promotes bacterial translocation[36]. Bacteria colonizing the gastrointestinal tract can translocate from the intestinal lumen to the bloodstream, and this may cause both systemic infection and/or infections in distant organs, such as mesenteric lymph nodes, spleen and liver[9]. Intestinal I/R injury does not only result in bacterial translocation, but also promotes an increase in plasma endotoxin level[6,37]. Our study also demonstrated a significant increase in plasma endotoxin level parallel to disruption of intestinal tissue in the intestinal I/R group when compared to the control group. Bacterial translocation and increased levels of endotoxin in the circulation may initiate a systemic inflammatory response, and the secretion and activation of inflammatory mediators, including cytokines[38] and metabolites of arachidonic acid[39]. An increase in intestinal permeability, and the deleterious effect of endotoxin in non-steroidal anti-inflammatory drug (NSAID)-induced ulcers has also been observed in experimental NSAID-induced enteropathy[40]. Gut decontamination decreases plasma endotoxin levels, and attenuates the systemic injury in intestinal I/R in rats[11]. A beneficial effect of antibiotics was also observed in NSAID-induced enteropathy[41,42].

The results of our study demonstrated that glutamine pretreatment prevented the increase in intestinal permeability during intestinal I/R, which has been reported previously[6,43]. However, glutamine pretreatment itself did not change the intestinal permeability significantly when compared to that in the control group. It has previously been shown that administration of glutamine-enriched nutritional support protects the bowel from injury due to abdominal irradiation[44], chemotherapy[45] and sepsis[46].

Glutamine pretreatment also caused a decrease in plasma endotoxin level, which did not reach statistical significance in our study. Contrary to our result, Wu *et al* have reported that an increase in plasma endotoxin level due to intestinal I/R can be reduced by Gln-supplemented total parenteral nutrition[6]. However, our study differed from that of Wu *et al* regarding the dose, route and timing of Gln administration. Furthermore, Gln was delivered in a total parenteral nutrition solution containing various amino acids and lipids in the study of Wu *et al*. Entrance of ^{51}Cr-EDTA and endotoxin to the systemic circulation from the intestinal lumen may be driven by different mechanisms, which may also explain differential effects of Gln on the above-mentioned parameters. The possibility that colonic epithelial cells contain specific transport systems for endotoxin has been reported[47]. Intestinal I/R disrupted intestinal tissue significantly. Gln pretreatment did not prevent histopathological disruption. It has also been observed that there is no correlation between histopathological alterations and intestinal permeability during recovery after hemorrhagic shock which is accepted as a model of intestinal I/R[30].

In conclusion, although Gln pretreatment reversed increased intestinal permeability, it did not prevent an increase in plasma endotoxin levels or histopathological alterations in intestinal I/R. Further studies are necessary to clarify the effects of different doses and administration periods of Gln on plasma endotoxin levels and histopathological changes in intestinal I/R.

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COMMENTS

Background

Impairment of microcirculation during I/R in the gastrointestinal tract may diminish intestinal mucosal integrity and cause an increase in intestinal permeability. Increased plasma endotoxin levels after I/R are a major threat to many surgical patients. Different Gln treatments are known to have protective effects on mucosal integrity in intestinal I/R.

Research frontiers

Injury to the intestinal barrier results in an increase in permeability to intraluminal substances. Correlation between morphological alterations and the degree of increased intestinal permeability is uncertain. Recent studies have demonstrated that morphological alterations after intestinal mucosal injury cannot reflect the function of the intestinal barrier.

Innovations and breakthroughs

Recent studies suggest that gastrointestinal epithelial cells contain specific transport systems for lipopolysaccharides.

Applications

Intestinal permeability can be measured by many different *in vitro* and *in vivo* methods. Endotoxin passing through the intestinal mucosa into the circulation indicates loss of the intestinal barrier function. Measurement of plasma endotoxin level ensures the assessment of real pathogenic factors that arise in the systemic circulation as a result of intestinal mucosal injury.

Terminology

Bacterial translocation: indigenous bacteria that colonize the gastrointestinal tract can cross the epithelial mucosa to infect distant organs. Under normal conditions, the epithelial lining prevents the escape of these bacteria from the gut lumen. Intestinal permeability: relates to the properties and function of the epithelial barrier that enables unmediated passage of substances through the intestinal mucosa. The use of intestinal permeability tests for screening of intestinal disease and assessment of treatment efficacy, to understand the normal intestinal physiology and pathogenesis of disease, is described and reviewed. There is now a need for research into the basic mechanisms of regulatory control of the intestinal barrier function.

Peer review

Peer reviewers considered this to be a very interesting paper with a great deal of potential clinical benefit. These basic results may be of value in clinical applications in organ or cellular transplantion.

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RAPID COMMUNICATION

Single-center study comparing computed tomography colonography with conventional colonoscopy

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Author contributions: Dr Tucker G analyzed the results; Professor Roberts-Thomson I wrote the paper largely; Professor Roberts-Thomson I and Mr Hewett P performed the colonoscopy; Dr Sebben R and Dr Khoo E are consultant radiologists; Ms Marker J and Dr Clapton W represented the Department of Health and Mr Cheung P was a clinical research coordinator
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Abstract

AIM: To compare the results from computed tomography (CT) colonography with conventional colonoscopy in symptomatic patients referred for colonoscopy.

METHODS: The study included 227 adult outpatients, mean age 60 years, with appropriate indications for colonoscopy. CT colonography and colonoscopy were performed on the same day in a metropolitan teaching hospital. Colonoscopists were initially blinded to the results of CT colonography but there was segmental unblinding during the procedure. The primary outcome measures were the sensitivity and specificity of CT colonography for the identification of polyps seen at colonoscopy (i.e. analysis by polyp). Secondary outcome measures included an analysis by patient, extracolonic findings at CT colonography, adverse events with both procedures and patient acceptance and preference.

RESULTS: Twenty-five patients (11%) were excluded from the analysis because of incomplete colonoscopy or poor bowel preparation that affected either CT colonography, colonoscopy or both procedures. Polyps and masses (usually cancers) were detected at colonoscopy and CT colonography in 35% and 42% of patients, respectively. Of nine patients with a final diagnosis of cancer, eight (89%) were identified by CT colonography as masses (5) or polyps (3). For polyps analyzed according to polyp, the overall sensitivity of CT colonography was 50% (95% CI, 39%-61%) but this increased to 71% (95% CI, 52%-85%) for polyps $\geqslant$ 6 mm in size. Similarly, specificity for all polyps was 48% (95% CI, 39%-58%) increasing to 67% (95% CI, 56%-76%) for polyps $\geqslant$ 6 mm. Adverse events were uncommon but included one colonic perforation at colonoscopy. Patient acceptance was high for both procedures but preference favoured CT colonography.

CONCLUSION: Although CT colonography was more sensitive in this study than in some previous studies, the procedure is not yet sensitive enough for widespread application in symptomatic patients.

Key words: Colorectal polyps; Colorectal cancer; Computed tomography colonography; Colonoscopy

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INTRODUCTION

CT colonography is a newer radiological procedure that might be suitable for screening for colorectal lesions in symptomatic and asymptomatic individuals[1-6]. An obvious consideration is the sensitivity, specificity and accuracy of the investigation in different patient populations. A second consideration is the frequency of colorectal abnormalities on CT colonography that will require subsequent colonoscopy. Ideally, this percentage should be relatively low. Yet a third consideration is the cost of the procedure in money and time and the frequency of minor and more serious complications.

In previous studies that have compared CT colonography

with colonoscopy, the sensitivity, specificity and accuracy of CT colonography has shown substantial variation. In one large screening study in asymptomatic adults, CT colonography had a similar sensitivity to colonoscopy for the detection of polyps greater than 6 mm in diameter[7]. In contrast, studies in symptomatic patients have shown a sensitivity for the detection of cancers of approximately 75% and lower sensitivities for the detection of polyps[8,9]. This variation may reflect differences in bowel preparation, colonic distension, CT scanners, collimation, software, scan evaluation and use of fecal tagging[10-13]. In this study, the comparison of CT colonography with colonoscopy included the routine use of hyoscine butylbromide (Buscopan) during CT colonography, unblinding of the results of CT colonography during colonoscopy and repeat colonoscopy for discrepant findings.

MATERIALS AND METHODS

The study included 227 patients with appropriate indications for colonoscopy. Symptoms included rectal bleeding in 77 patients, abdominal pain in 56 and a change in bowel habit in 41. In addition, some patients had a family history of colorectal cancer (51), previous colonic polyps (43) and a recent positive fecal occult blood test (30). Exclusion criteria included inflammatory bowel disease and major coexisting medical disorders. Patients had a mean age of 60 years with an age range of 25 to 85 years. There were similar numbers of men (51%) and women (49%). All patients gave informed written consent and the study was approved by the local institutional Ethics Committee in 2003.

CT colonography and colonoscopy were performed on the same day. The type of bowel preparation was determined by the colonoscopist but most patients had Picolax, Colonlytely or both preparations. CT colonography was performed with a Toshiba multislice helical CT scanner with 2 mm collimation that was reconstructed into intervals of 1.0-1.5 mm. Colonic distension was achieved by insufflation of carbon dioxide and the use of intravenous Buscopan (20 mg). Patients were scanned in both supine and prone positions during a single breath hold (average scanning time, 25 s). With this technique, the radiation dose is lower than that of a barium enema X-ray. Images obtained with a Compaq® PC using ColonScreen (Voxcar Ltd., Edinburgh) were read in 2-dimensional format with use of a targeted 3-dimensional format when necessary. The CT images were reported by one of three radiologists within 60 min of completion of the scan. All had received previous training in CT colonography and had been reporting CT colonography on a routine basis prior to the study. The adequacy of the bowel preparation was recorded and the presence and size of polyps and masses were reported for eight segments (caecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon and rectum).

Colonoscopy was performed by a consultant gastroenterologist or colorectal surgeon. Patients were sedated with intravenous fentanyl and midazolam, sometimes supplemented with propofol. The colonoscope was passed to the caecum and then slowly withdrawn to the splenic flexure. At that point, CT colonography findings in the right colon were made available to the colonoscopist and colonoscopy was repeated if there were discrepant results. This also applied for CT colonography findings in the left colon after withdrawal of the colonoscope to the rectum. Masses and polyps detected at colonoscopy were reported in eight segments as above.

The quality of the bowel preparation was recorded on a scale of 1-5 using the system of Yee *et al*[14]. Patient discomfort with both CT colonography and colonoscopy were assessed by a questionnaire soon after CT colonography and during recovery after colonoscopy. Patients also completed a similar questionnaire 1 wk after the procedures that included a question on preference for CT colonography or colonoscopy. All adverse events were recorded.

Statistical analysis

Comparisons of CT colonography with colonoscopy were analyzed according to polyp and according to patient using Stata Version 9. In the more important analysis according to polyp, confidence intervals were produced using logistic regression and reflect an allowance for clustering of polyps within patients. Polyps were judged to be identical if they were located in the same or adjacent segments and if they were of similar size (± 50%). In the analysis according to patient, exact confidence intervals were calculated by the Stata contributed program "diagt". Patients with at least one polyp identified by colonoscopy were a true positive if they had at least one polyp at CT colonography. It was not necessary for the lesions to be assessed as identical.

RESULTS

CT colonography was compared with colonoscopy in 202 of 227 patients. Twenty-five patients (11%) were excluded from the analysis because of incomplete colonoscopy or poor bowel preparation that largely affected CT colonography. Polyps and masses were detected at CT colonography and colonoscopy in 42% and 35% of patients, respectively.

Detection of cancer

Of the 9 patients with a final diagnosis of cancer, 8 were diagnosed at colonoscopy and confirmed by biopsy while 1 patient had cancer within a polyp that was diagnosed histologically. Cancers were located in the sigmoid colon (3), rectum (3), ascending colon (2) and splenic flexure (1). All of these lesions were seen at CT colonography but only 5 were considered as probable cancers. The remaining lesions were interpreted as polyps (3) and fecal material (1). Three additional patients were diagnosed with probable cancer at CT colonography but only 1 had a corresponding polyp at colonoscopy. Polyps and cancers detected at both CT colonography and colonoscopy are shown in Figure 1.

Detection of polyps

At colonoscopy, 163 polyps were detected in 69 patients. The most common sites were the sigmoid colon (30%), rectum (27%), transverse colon (22%) and ascending colon (12%). Histologically, the majority of polyps were

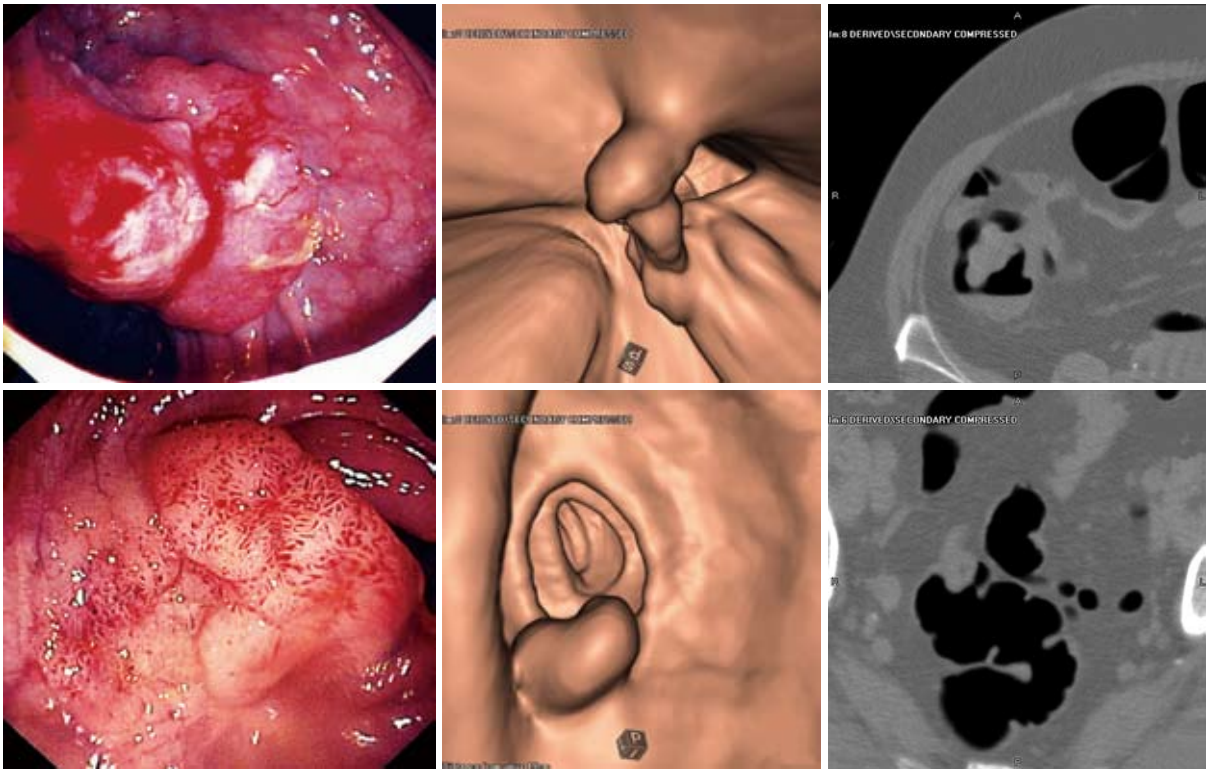

Figure 1 The figure shows the endoscopic appearance and images at CT colonography (3-D and 2-D) for 2 colonic lesions. The upper panel shows a cancer in the ascending colon while the lower panel shows a large tubulovillous adenoma at the rectosigmoid junction.

adenomatous (67%) while the remainder were hyperplastic, serrated or unspecified. In the analysis according to polyp, the sensitivity, specificity and accuracy of CT colonography for the detection of polyps of various sizes is shown in Table 1. The sensitivity for all polyps was 50% but this increased to 71% for polyps $\geqslant$ 6 mm in size. In the analysis according to patient, CT colonography had an overall sensitivity, specificity and accuracy of 62% (95% CI, 50%-74%), 76% (95% CI, 67%-83%), and 71% (95% CI, 64%-77%), respectively. For polyps $\geqslant$ 6 mm analyzed by patient, sensitivity, specificity and accuracy were 78% (95% CI, 58%-91%), 82% (95% CI, 76%-88%) and 82% (95% CI, 76%-87%). In relation to the sensitivity of colonoscopy, one polyp was detected by CT colonography that was missed at the initial colonoscopy but detected by repeat colonoscopy.

Extracolonic findings

Data on extracolonic findings were available in 225 of 227 patients (99%). One patient had a renal mass and was subsequently diagnosed with renal cell cancer. Other findings of potential clinical significance included renal stones (7%), gallbladder stones (6%), adrenal masses (4%), ovarian cysts (3%), non-cystic liver lesions (2%), small aortic aneurysms (2%), lung nodules (1%) and calcific chronic pancreatitis (1%).

Bowel preparation at CT colonography

Only 33% of patients had a clean bowel (grade 1) while 62% had "pools of liquid" (grade 2) and 5% had "solid and liquid" or "collections of solid feces" (grades 3 and 4). Patients categorized as "impossible" (grade 5) were excluded from the study.

Adverse events

These were reported by 4 patients after CT colonography and included nausea (2), dizziness (1) and significant abdominal pain (1). Adverse events after colonoscopy included rectal bleeding (1) and moderate or severe abdominal pain (2). One of these patients was subsequently diagnosed with colonic perforation and treated by resection of the sigmoid colon. There were no postoperative complications. Other recorded complications during colonoscopy included hypoxia (1), hypertension (1) and bradycardia (1).

Patient acceptance and preference

Selected results from a questionnaire administered 1 wk after the procedures are included in this report. The questionnaire was completed by 195 of 202 patients (96%). The experience of CT colonography and colonoscopy was "better than expected" or "as expected" in 84% and 94% of patients, respectively. Overall, 87% of patients were "very satisfied" or "satisfied" with CT colonography while 90% were "very satisfied" or "satisfied" with colonoscopy. Furthermore, 89% of patients indicated that they "definitely would" or were "most likely" to have repeat CT colonography compared to 96% for colonoscopy. These differences were not statistically significant. However, when asked to choose between CT colonography and colonoscopy for a repeat procedure, 61% chose CT colonography and 39% chose colonoscopy ($P = 0.005$, Chi-square test).

DISCUSSION

CT colonography, also called virtual colonoscopy, is an

Table 1 Sensitivity, specificity and accuracy of CT colonography when analyzed by polyp (%)

Polyp size	*n*	Sensitivity	Specificity	Accuracy
< 6 mm	125	42 (30-55)	63 (52-73)	54 (46-62)
6-10 mm	27	78 (52-92)	75 (65-83)	75 (66-83)
> 10 mm	11	62 (39-81)	86 (77-92)	82 (73-89)
All polyps	163	50 (39-61)	48 (39-58)	49 (42-56)
⩾ 6 mm	38	71 (52-85)	67 (56-76)	68 (59-76)

Values in brackets are 95% confidence intervals that reflect an allowance for clustering of polyps within patients.

evolving technology that may have a role in screening for colorectal polyps and cancer. However, using colonoscopy as the gold standard, concordance between the two investigations has varied widely in different studies. In relation to the patient population, better results have been achieved in screening studies in asymptomatic individuals than in patients with symptoms. This may reflect the younger age and better health of asymptomatic individuals who are more able or more motivated to comply with bowel preparation procedures. In any event, the quality of the bowel preparation is more important for CT colonography than for colonoscopy. In the present study, the adequacy of the bowel preparation was recorded by the reporting radiologist. Only 33% of patients had a "clean bowel" while the remainder had either "pools of liquid" (62%) or "solid and liquid" (5%). Whether bowel images can be improved by modified preparation protocols[15] or by "electronic cleansing"[16] remains unclear. Intravenous Buscopan is not routinely used during CT colonography but does enhance colonic distension[17].

Other factors that may contribute to variable results from CT colonography include the type and settings of CT scanners, the use of fecal tagging, the mode of imaging and the experience of the reporting radiologist. In one meta-analysis[10], better results were achieved with scanners with multiple detectors, thinner collimation and the standard use of three-dimensional ("fly-through") technology. Fecal tagging using oral contrast did not appear to be a relevant factor and has the potential to impair the quality of colonoscopy. When images are reported by different radiologists, variation has ranged from "minimal"[7] to "substantial"[18], apparently independent of previous experience with the technique. There are also potential biases in comparative studies of this type with over-reporting in the investigation under study, specifically CT colonography.

The experience of both CT colonography and colonoscopy was widely accepted by patients. Although responses to specific questions about each technique showed a trend in favour of colonoscopy, more patients chose CT colonography when given a choice between the two procedures. These responses can be influenced by the structure of the questions but might also reflect real concerns about sedation, potential complications and the degree of "invasiveness". In this study, complications were uncommon but one patient had a colonic perforation that required surgery. This complication has an overall frequency of approximately 1 in 1000 colonoscopies[19,20] but can also occur after CT colonography, perhaps with a frequency of 1 in 1700 procedures[21].

In this study, CT colonography was more sensitive than in previous studies in symptomatic patients[8,9]. For example, CT colonography identified cancers as either masses or polyps in 8 of 9 patients (89%) in this study and in 6 of 8 (75%)[8] and 7 of 9 (78%)[9] in previous studies. For polyps analyzed by polyp, the overall sensitivity for detection of polyps was 50% in this study increasing to 71% for polyps 6 mm in size or greater. In previous studies, sensitivities for polyp detection (⩾ 6 mm) were reported as 32%[8] and 47%[9]. The possibility that inaccurate localisation of polyps at colonoscopy contributed to low sensitivity rates seemed unlikely as sensitivities for polyps ⩾ 6 mm analyzed by patient were only marginally higher (78%) than those analyzed by polyp (71%).

Criteria for the introduction of new technologies into clinical practice are difficult to establish. For example, issues such as sensitivity and specificity need to be considered in relation to direct and indirect costs, complications and the proportion of patients who will be referred for further investigation. In the present study, CT colonography identified polyps or masses in 42% of patients. This is not a cost-effective option if all patients with abnormalities are referred for colonoscopy[22]. However, CT colonography becomes increasingly cost-effective if colonoscopy is restricted to lesions that are ⩾ 6 mm or ⩾ 10 mm in size. Such recommendations, if introduced, would invite a new series of questions related to the frequency of repeat scans and the mutagenic effects of accumulating doses of radiation[23-25]. Although small polyps have a low frequency of features of advanced neoplasia, there is only limited data on the natural history of small polyps and the identification of those polyps that will eventually evolve into cancer[7,26].

Although CT colonography is not yet ready for widespread clinical application, it is likely that results will improve with better bowel preparation, technical developments and increasing familiarity with the technique. Results from this study highlight the importance of bowel preparation although, in the future, it may be possible to reliably differentiate fecal material from polyps using fecal tagging[10], "electronic cleansing" of colonic fluid[16] or contrast-enhanced studies[27]. Other helpful developments may also include new fecal subtraction algorithms[28], workstation modifications to facilitate polyp detection[29], improved software and new training programs for radiologists[30].

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COMMENTS

Background

It is not yet clear whether computed tomography (CT) colonography will be widely adopted for the detection of colonic polyps and cancer. Current issues include

the sensitivity and specificity of the investigation, direct and indirect costs and the longer-term effects of radiation.

Research frontiers

This study indicates that residual liquid and solid material after bowel preparation limits the accuracy of the procedure. Whether this can be overcome by modified laxative preparations, fecal tagging or "electronic" bowel cleansing remains unclear.

Innovations and breakthroughs

In this study in symptomatic patients, CT colonography was more sensitive for the detection of polyps than in some previous studies. This study included the routine use of intravenous Buscopan but the study was not designed to determine whether this modification improved either sensitivity or specificity.

Applications

There is widespread interest in screening for colon cancer in symptomatic and asymptomatic individuals. Colonography, either using CT or magnetic resonance imaging, may play a central role if there are further improvements in sensitivity and specificity.

Peer review

The paper by Roberts-Thomson IC *et al* is interesting. The selection of patients, sample size and the overall design of the study are fair, the results adequate to provide clinical evidence and to support valid conclusions.

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RAPID COMMUNICATION

Impact of alanyl-glutamine dipeptide on severe acute pancreatitis in early stage

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Abstract

AIM: To evaluate the therapeutic effect of alanyl-glutamine dipeptide (AGD) in the treatment of severe acute pancreatitis (SAP) in early and advanced stage.

METHODS: Eighty patients with SAP were randomized and received 100 mL/d of 20% AGD intravenously for 10 d starting either on the day of (early treatment group) or 5 d after (late treatment group) admission. Groups had similar demographics, underlying diseases, Ranson score, Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE Ⅱ) score, and Balthazar's computed tomography (CT) score at the beginning of the study and underwent similar other medical and nutritional management.

RESULTS: The duration of acute respiratory distress syndrome (2.7 ± 3.3 d *vs* 12.7 ± 21.0 d, $P < 0.01$), renal failure (1.3 ± 0.5 d *vs* 5.3 ± 7.3 d, $P < 0.01$), acute hepatitis (3.2 ± 2.3 d *vs* 7.0 ± 7.1 d, $P < 0.01$), shock (1.7 ± 0.4 d *vs* 4.8 ± 3.1 d, $P < 0.05$), encephalopathy (2.3 ± 1.9 d *vs* 9.5 ± 11.0 d, $P < 0.01$) and enteroparalysis (2.2 ± 1.4 d *vs* 3.5 ± 2.2 d, $P < 0.01$) and hospital stay (28.8 ± 9.4 d *vs* 45.2 ± 27.1 d, $P < 0.01$) were shorter in the early treatment group than in the late treatment group. The 15-d APACHE Ⅱ score was lower in the early treatment group than in the late treatment group (5.0 ± 2.4 *vs* 8.6 ± 3.6, $P < 0.01$). The infection rate (7.9% *vs* 26.3%, $P < 0.05$), operation rate (13.2% *vs* 34.2%, $P < 0.05$) and mortality (5.3% *vs* 21.1%, $P < 0.05$) in the early treatment group were lower than in the late treatment group.

CONCLUSION: Early treatment with AGD achieved a better clinical outcome in SAP patients.

Key words: Severe acute pancreatitis; Alanyl-glutamine dipeptide; Clinical study

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INTRODUCTION

Acute pancreatitis (AP) contributes to thousands of annual hospital admissions, of which severe acute pancreatitis (SAP) accounts for 10%-20%[1-3]. Despite considerable improvement in the treatment, the mortality of SAP still ranges between 10%-15%[2-5].

The course of SAP tends to be prolonged and the patients usually are hypermetabolic and high protein catabolic due to systemic inflammatory response syndrome (SIRS) induced by acute local inflammatory process and subsequent vital-organ dysfunction[6]. Thus, if nutritive support is not appropriately administrated to match rapidly increased demand in the treatment of SAP, the patients consequently come down with metabolic disorder and nutrition deficiency, which is considered to increase mortality due to impaired immune function, increased risk of infections and intractable vital-organ failure.

In recent years, research showed a conditional deficiency of glutamine would be an independent predictive factor for a poor outcome and its correction improved survival by restoring cellular protective mechanisms, improving immune function and resistance of the gut barrier to hypoperfusion, metabolic stress and subsequent bacterial translocation, and decreasing the risk of infection in critical illness[7-10]. Since free glutamine is instable in solution, intravenous administration is limited[7]. Alanyl-glutamine

dipeptide (AGD), however, can be taken *via* vein and hydrolyzed into alanine and glutamine in circulation as a substitute[7]. Presently AGD supplement in parenteral nutrition is a worth-trying approach and an evidence-based recommendation in the management of SAP[11], but there has been no study describing an optimal protocol of AGD administration. Our study aims to evaluate the favorable effects of early supplement with AGD in the treatment of SAP.

MATERIALS AND METHODS

Patient selection

In this study, the diagnostic criteria[12] formulated for SAP at the Bangkok World Congress of Gastroenterology 2002 in Thailand was employed. All of the patients, who had been diagnosed with SAP and admitted to our hospital within 72 h after onset of symptoms, were included. The patients who had histories of trauma, operation or prior treatment with AGD were excluded, and the patients who died within 5 d after admission were also rejected.

Methods

In this study, 80 identified SAP patients who were admitted to West China Hospital of Sichuan University from May 2004 to March 2005 were randomized and treated with 100 mL/d of 20% AGD intravenous infusion for 10 d (SSPC No. SF1505) starting either on the day of (early treatment group) or 5 d after (late treatment group) admission.

Upon admission, all of the patients were treated with intensive care, oxygen inhalation, intermittent gastrointestinal decompression, and fluid infusion. Prophylactic antibiotics were used for 7-14 d. H2 receptor antagonist or proton pump inhibitor agent was given for 7 d; the acid-base balance and the electrolyte balance were maintained. When patients developed respiratory failure, the respirator was employed to assist respiration. When hypoalbuminaemia occurred, 20% human serum albumin 50 mL was used daily until the serum albumin was recovered to normal, and fat emulsion was also given for 14 d.

The following parameters were measured: 24-h APACHE Ⅱ score and initial Balthazar's CT score, 15-d APACHE Ⅱ score, incidence and duration of complications including acute respiratory distress syndrome (ARDS), renal failure, acute hepatitis, encephalopathy and enteroparalysis, infection rate, hospital stay, operation rate and mortality.

Hospital stay: Hospital stay was defined as the duration from hospital admission to discharge. The duration of hospital rehabilitation due to cholecystectomy was not taken into account in this study, although cholecystectomy was regarded as a promising treatment to prevent recurrent pancreatitis.

Operation rate: Surgical intervention was performed if infected pancreatic necrosis, or pancreatic abscess, or (per)pancreatic hemorrhage or (per)pancreatic pseudocyst was identified or if the patient did not respond to intensive care treatment.

Table 1 Baseline in the two groups

Baseline	Late treatment group (n = 38)	Early treatment group (n = 38)
Sex (Male/Female)	21/17	22/16
Age (mean ± SD, yr)	47.5 ± 12.6 (22-76)	46.9 ± 12.8 (27-74)
Etiology, n (%)		
Gallstones	20 (52.6)	16 (42.1)
Alcohol abuse	2 (5.3)	7 (18.7)
Hyperlipidemia	7 (18.4)	9 (23.7)
Idiopathic	9 (23.7)	6 (15.8)
48-h Ranson score (mean ± SD)	4.5 ± 1.7	4.8 ± 1.6
24-h APACHE Ⅱ score (mean ± SD)	10.8 ± 3.5	10.2 ± 3.1
CT score (mean ± SD)	5.8 ± 2.3	5.9 ± 2.4

APACHE Ⅱ: Acute Physiology and Chronic Heath Evaluation Ⅱ; CT: Computed Tomography.

Statistical analysis

Data were expressed as mean ± SD or percentage. Data in normal distribution was analyzed using t test; data in non-normal distribution was analyzed using Wilcoxon rank sum test. Categorical data was analyzed using Chi-square test. $P < 0.05$ was considered statistically significant.

The medical ethics committee of West China Hospital at Sichuan University approved this study. All patients gave their informed consent, and the study was conducted according to the recent principles of the Declaration of Helsinki (World Medical Association, 2000).

RESULTS

Four patients including 1 death within 24 h after admission and 1 death on the 5th day after admission in the early treatment group and 2 deaths within 24 h in the late treatment group withdrew from the study, which were not included in any of the analyses. Therefore, there were 38 patients in the early treatment group and 38 in the late treatment group.

Baseline

There were no statistical differences between the two groups in sex, age and etiology ($P > 0.05$, Table 1), and in the 48-h Ranson score, 24-h APACHE Ⅱ score and CT score in the initial stage of hospitalization ($P > 0.05$, Table 1).

Complications

There were no statistical differences between the two groups in the incidences of ARDS, renal failure, shock, acute hepatitis, encephalopathy and enteroparalysis ($P > 0.05$), but the duration of ARDS, renal failure, acute hepatitis, encephalopathy and enteroparalysis was shorter in the early treatment group than in the late treatment group ($P < 0.01$), and the duration of shock was also shorter in the early treatment group ($P < 0.05$) (Table 2).

Prognosis

The 15-d APACHE Ⅱ score was lower in the early treatment group than in the late treatment group ($P < 0.01$).

Table 2 Incidence and duration of complications in the two groups (*n*, %) (mean ± SD)

Complications	Late treatment group (*n* = 38)		Early treatment group (*n* = 38)	
	Cases (%)	Duration (d)	Cases (%)	Duration (d)
ARDS	18 (47.4)	12.7 ± 21.0	21 (55.3)	2.7 ± 3.3[b]
Renal failure	9 (23.7)	5.3 ± 7.3	7 (18.4)	1.3 ± 0.5[b]
Acute hepatitis	19 (50.0)	7.0 ± 7.1	17 (44.7)	3.2 ± 2.3[b]
Shock	6 (15.8)	4.8 ± 3.1	9 (23.7)	1.7 ± 0.4[a]
Encephalopathy	4 (10.5)	9.5 ± 11.0	4 (10.5)	2.3 ± 1.9[b]
Enteroparalysis	27 (71.1)	3.5 ± 2.2	29 (76.3)	2.2 ± 1.4[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* late treatment group; ARDS: acute respiratory distress syndrome.

Table 3 Fifteen-day APACHE Ⅱ score, hospital stay (d), infection, operation and mortality in the two groups

Prognosis	Late treatment group (*n* = 38)	Early treatment group (*n* = 38)
15-d APACHE Ⅱ score (mean ± SD)	8.6 ± 3.6	5.0 ± 2.4[b]
Hospital stay (d, mean ± SD)	45.2 ± 27.1	28.8 ± 9.4[b]
Infection, *n* (%)	10 (26.3)	3 (7.9)[a]
Operation, *n* (%)	13 (34.2)	5 (13.2)[a]
Deaths, *n* (%)	8 (21.1)	2 (5.3)[a]

[a]$P < 0.05$, [b]$P < 0.01$.

The infection rate, operation rate and mortality were also lower in the early treatment group ($P < 0.05$). The hospital stay was shorter in the former group than in the latter group ($P < 0.01$) (Table 3).

DISCUSSION

In the early stage of SAP, the patients tend to be hypermetabolic due to occurrence of SIRS and subsequent multiple organ dysfunction syndromes (MODS), resulting in the greatly increased demand for nutrition[13-15]. In the late stage, the demand for nutrition increases continuously due to infection, resulting from intestinal bacterial translocation and immunosuppression. Thus, insufficient nutritive support inevitably leads to nutrition deficiency in SAP patients[16].

When a nutritional deficiency arises in critical illness including SAP, glutamine, which is very abundant and readily synthesized under most situations, tends to be a conditional depletion[17]. The low concentration of plasma glutamine was found to be an independent predictive factor for a poor outcome in critical illness[18].

AGD was shown to improve clinical outcome of SAP[11,16]. In this study, we treated SAP patients with 100 mL/d of 20% AGD infusions intravenously for 10 d to compare the effects of AGD between the two groups and study its optimal protocol. The baseline data showed no significant difference between the two groups ($P > 0.05$). APACHE Ⅱ score, the parameter for predicting and monitoring the development of local and systemic complications of SAP, was evaluated on the 15th day after admission, which was lower in the early treatment group than in the late treatment group ($P < 0.01$). The duration of ARDS, renal failure, acute hepatitis, shock, encephalopathy and enteroparalysis were also shorter in the early treatment group than in the late treatment group, as was lower mortality ($P < 0.05$). These might result from a possible consequence of early suppression of inflammatory response and restoring cellular protective mechanisms by early AGD supplementation, which is associated with mediating anti-inflammatory/immunologic factors[19] and antioxidant/inducible nitric oxide synthase (iNOS)[20], decreasing the level of TNF-alpha and interleukin-8 in mononuclear cell[21,22] and C-reactive protein in serum[23]. In this study, the hospital stay was also shorter in the early treatment group ($P < 0.05$), which might be correlated to the shorter duration of complications.

Intestinal tract, the greatest "storeroom" of microorganisms, also is one of the most possible injury organs besides pancreas itself[24]. Intestinal bacterial translocation resulting from the impaired mucosal barrier, microorganism barrier, chemical barrier and immune barrier is an early event[25] and the main cause of infection in SAP[26]. The infection rate of SAP was 30%-50%[27-30], leading to a mortality of 40%-80%[31,32]. Glutamine is used as a major fuel and nucleotide substrate for rapidly dividing cells such as intestinal mucosal cells and the gut-associated immunocytes[33-37]. Glutamine can prevent atrophy of the intestinal epithelial cells through HSP 70 generation[38] and improve the intestinal immune barrier[39-41]. A meta-analysis by Novak F *et al*[42] revealed that glutamine could reduce the infectious morbidity (RR 0.84, 95% CI: 0.68-1.03) and mortality (RR 0.76, 95% CI: 0.56-0.98) in critical illness. Our study showed that early treatment with AGD in SAP patients could shorten the duration of enteroparalysis ($P < 0.01$) and reduce the infection rate ($P < 0.05$). This result may indicate that early nutrition with AGD in SAP patients could timely protect and restore the function of intestinal tract, shorten the duration of enteroparalysis, lower the risk of bacterial translocation, thus reducing the infection rate.

In conclusion, early treatment with AGD yields a better clinical outcome in SAP patients by reducing the duration of vital-organ dysfunction, decreasing the risk of infection and operation, and lowering the mortality.

COMMENTS

Background

Severe acute pancreatitis (SAP) is a hypermetabolic disease. Appropriate nutrition support is essential to the management of SAP. In recent years, the supplement of glutamate, the most abundant free amino acid playing vital roles in the body, has been shown to improve survival rate. Presently, the supplement of alanyl-glutamine dipeptide, as a substitute of glutamate which is stable in circulation, is a promising and worth-trying approach.

Research frontiers

Besides nutritional management of SAP, restoring an optimized immune system plays a role in improving survival rate. Previous pilot studies of alanyl-glutamine dipeptide (AGD) supplementation in nutritive support have revealed good outcome by restoring cellular protective mechanisms, improving the immune function and lowering the infection rate. Although this treatment was recommended by evidence-based studies, this treatment principle has not yet been systematically applied and further study is still needed in this field.

Innovations and breakthroughs

The optimal protocol for the AGD treatment is not yet available in published

studies. Early AGD treatment in SAP is a breakthrough in this study, which reveals for the first time that early AGD treatment achieved a better clinical outcome in SAP patients.

Applications

As free glutamine is instable in solution, intravenous administration is limited. AGD, however, can be given *via* vein and hydrolyzed into alanine and glutamine in circulation as a substitute. This study showed that early treatment with AGD achieved a better clinical outcome in SAP patients. This treatment can be applied in the management of SAP patients.

Terminology

Severe acute pancreatitis is a common acute abdominal disorder, characterized by various degrees of necrosis of pancreatic parenchyma together with local and systemic complications, such as SIRS and multiple organ dysfunction syndromes. Alanyl-glutamine dipeptide is an important component of parenteral nutrition ingredients with its molecular formula ingredient as N (2)-L-alanyl-L-glutamine. SIRS is a clinical response to one of the nonspecific insults caused by ischemia, inflammation, trauma, infection, or a combination of several insults, which was defined by a journal in 1992 and described as occurrence of two or more clinical symptoms of fever or hypothermia, tachypnea, tachycardia, and leukocytosis.

Peer review

AGD supplementation has been shown to be effective by previous studies. In this pilot study, the authors focused on the clinical effects of early treatment with AGD comparing with late treatment, which shows early AGD treatment indicates a better clinical outcome. Further researches are needed to explore its mechanism.

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RAPID COMMUNICATION

Comparison of real-time polymerase chain reaction with the COBAS Amplicor test for quantitation of hepatitis B virus DNA in serum samples

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Abstract

AIM: To compare the clinical performance of a real-time PCR assay with the COBAS Amplicor Hepatitis B Virus (HBV) Monitor test for quantitation of HBV DNA in serum samples.

METHODS: The reference sera of the Chinese National Institute for the Control of Pharmaceutical and Biological Products and the National Center for Clinical Laboratories of China, and 158 clinical serum samples were used in this study. The linearity, accuracy, reproducibility, assay time, and costs of the real-time PCR were evaluated and compared with those of the Cobas Amplicor test.

RESULTS: The intra-assay and inter-assay variations of the real-time PCR ranged from 0.3% to 3.8% and 1.4% to 8.1%, respectively. The HBV DNA levels measured by the real-time PCR correlated very well with those obtained with the COBAS Amplicor test (r = 0.948). The real-time PCR HBV DNA kit was much cheaper and had a wider dynamic range.

CONCLUSION: The real-time PCR assay is an excellent tool for monitoring of HBV DNA levels in patients with chronic hepatitis B.

Key words: COBAS Amplicor test; Hepatitis B virus; Viral DNA; Real-time PCR

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INTRODUCTION

It is estimated that 350 million individuals are chronically infected with hepatitis B virus (HBV) worldwide, and about one-third of these live in China[1]. HBV infection can cause chronic hepatitis, cirrhosis and hepatocellular carcinoma[2]. Among patients with active viral replication, cirrhosis will develop in 15%-20% within 5 year[3], and 70-90 percent of cases of hepatocellular carcinoma occur against a background of cirrhosis[4]. Quantitation of HBV DNA in serum or plasma has been an important tool in identifying individuals with active hepatitis B, to monitor the efficiency of antiviral treatment, and to predict whether the treatment will be successful[5-9].

Several commercial molecular assays have been developed for quantitation of HBV DNA in serum or plasma samples. One of these assays is the COBAS Amplicor HBV Monitor, which is based on the amplification of DNA targets by the use of HBV-specific primers. Other methods, such as the VERSANT HBV DNA 3.0 assay, which is based on branched-DNA signal amplification, and Hybrid Capture Ⅱ, which is based on hybridization of a chemiluminescent probe, are also routinely used in diagnostic laboratories[10,11]. However, the costs of these assays are too high to be used in developing countries such as China, and in these countries the HBV burden is much heavier than in developed countries.

Recently, quantitative real-time PCR has been used to detect and quantify HBV levels in serum or plasma samples. This assay is based on the relationship between the initial DNA target levels and the threshold cycle (Ct) of the amplification products. The Ct value is smaller when the initial DNA target level is higher. Several evaluation studies have shown that real-time PCR has a higher sensitivity, a broader dynamic range, and an accurate quantitation of HBV DNA compared to those of the existing commercial methods[12-15].

The Fosun real-time PCR HBV kit is a commercial

assay for quantitation of serum HBV DNA levels based on TaqMan PCR technology. It has been approved by the State Food and Drug Administration (SFDA) of China for *in vitro* diagnostic use. This assay is widely used in Chinese laboratories for quantitative detection of serum HBV levels in patients with HBV infection. However, the clinical performance of this real-time PCR assay has not been compared with that of commercial assays routinely used in the United States and Europe. The aim of this study was to evaluate the clinical performance of the Fosun real-time PCR HBV assay on the ABI 7500 sequence detector for the quantitative detection of HBV DNA in serum samples. The results were compared to those obtained with the same samples using the COBAS Amplicor HBV Monitor system.

MATERIALS AND METHODS

Patients and samples

Serum samples from 158 individuals, including 118 patients with chronic hepatitis B (46 genotype B, 65 genotype C, and 7 mixed genotype B and C), and 40 blood donors, were analyzed in parallel by the Fosun real-time PCR HBV kit (Fosun Diagnostics, Shanghai, China) and COBAS Amplicor HBV Monitor (Roche Diagnostics). All the patients were positive for hepatitis B surface antigen (HBsAg) for at least 6 mo, and were negative for antibodies to hepatitis A, C, D and E viruses. None of these patients had received any antiviral treatment at the time of serum collection.

An HBV reference serum from the National Center for Clinical Laboratories of China with a DNA level of 7.5×10^8 copies/mL was used to determine the linearity and detection limit of the real-time PCR. This reference serum was 10-fold serially diluted into a spectrum of reference samples with HBV DNA levels ranging from 7.5×10^1 to 7.5×10^8 copies/mL.

A set of HBV reference sera established by the Chinese National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), with HBV DNA concentrations ranging from < 1000 to 6.17×10^8 copies/mL of genotype C were used for standardization.

Real-time PCR assay

For Fosun real-time PCR, HBV DNA was extracted from 100 μL serum by using the extraction reagents included in the kit, in accordance with the manufacturer's instructions. The amplification was performed on an ABI 7500 sequence detector (Applied Biosystems, Foster City, CA, USA) by incubating the reaction mixture (50 μL) at 50℃ for 2 min, 94℃ for 5 min, followed by 40 cycles of PCR amplification at 93℃ for 30 s and 60℃ for 90 s. The Ct value was defined as the number of fractional cycles in which the fluorescence emitted by the reaction mixture exceeded a preset threshold, and marked the beginning of an exponential growth of the fluorescence signal. The results were analyzed with ABI Prism software (Applied Biosystems). A four-point external standard set was used to calculate the initial copy number of the samples. The dynamic range of Fosun real-time PCR HBV DNA kits, as stated by the manufacturer, is 4.2×10^2-7.5×10^8 copies/mL.

COBAS Amplicor assay

Amplicor tests were performed by using the COBAS Amplicor system and the AMPLICOR HBV Monitor assay according to manufacturer's instructions. The highly conserved HBV precore/core region and the internal standard DNA were amplified in the same well, but hybridized in separate wells. The amount of HBV DNA was calculated from the ratio of the HBV well to the internal standard well, and the copy number per milliliter was calculated from a standard curve. The dynamic range of the Amplicor test was 3×10^2-2×10^5 copies/mL.

Statistical analysis

Statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 13.0 for Windows; SPSS, Chicago, IL, USA). Correlation between the quantitative results from the two assays was determined with scatter plots and Spearman's coefficient analysis, after logarithmic transformation of data with skewed distribution. The Bland-Altman method was used to assess the agreement between the values obtained with the two assays[16].

RESULTS

Linearity and detection limit

The real-time PCR was able to detect seven (87.5%) of the eight dilutions of the reference serum. The tested values and the normal values of the dilutions were well matched ($r = 0.999$, $P < 0.001$). The converting formula was:

Log (HBV DNA level by real-time PCR) = 1.098 [Log (Reference HBV DNA level)] - 0.735

The real-time PCR was able to detect HBV DNA levels as low as 750 copies/mL, which is similar to the detection limit claimed by the manufacturer.

Inter-assay variation and accuracy of real-time PCR

To determine the dynamics and accuracy of the Fosun real-time PCR kit, the reference serum of the Chinese NICPBP was tested three times with kits with different lot numbers. Fosun real-time PCR kits detected all seven dilutions of the reference serum, and all the quantitative results were within the reference ranges (Table 1). The inter-assay variation ranged from 0.3% to 3.8%. The detection limit of the real-time PCR was 442 copies/mL.

Intra-assay variation and reproducibility of real-time PCR

For evaluation of the intra-assay variation of the Fosun real-time PCR kits, three samples with high, medium and low HBV titers were tested. Each sample was tested in 12 replicates. Table 2 shows the mean $\log_{10}$ (copies/mL) viral loads, SD and coefficient of variation (CV) of the samples. The viral load values from the Fosun real-time PCR assay were highly reproducible over the various HBV titers (CV, 1.4%-8.1%), especially for the samples with high and medium HBV titers, and the intra-assay CV was 1.4% and 2%, respectively.

Comparison of real-time PCR with COBAS Amplicor HBV Monitor

A total of 158 clinical serum samples were tested in

Table 1 Sensitivity of the Fosun real-time PCR HBV DNA assay and results of interassay testing

NICPBP references	Reference range (copies/mL)	Results (copies/mL)			Mean ($\log_{10}$ copies/mL)	SD ($\log_{10}$ copies/mL)	CV (%)
		Run 1	Run 2	Run 3			
L0	7.76×10^7 - 6.17×10^8	1.65×10^8	1.84×10^8	1.52×10^8	8.22	0.04	0.5
L1	1.48×10^7 - 1.18×10^8	9.21×10^7	1.01×10^8	9.23×10^7	7.98	0.02	0.3
L2	1.59×10^6 - 1.26×10^7	8.54×10^6	9.00×10^6	9.20×10^6	6.95	0.02	0.3
L3	1.66×10^5 - 1.32×10^6	7.06×10^5	6.13×10^5	9.55×10^5	5.87	0.10	1.7
L4	1.82×10^4 - 1.48×10^5	8.60×10^4	3.84×10^4	6.30×10^4	4.77	0.18	3.8
L5	1.51×10^3 - 1.23×10^4	1.07×10^4	6.20×10^3	8.82×10^3	3.92	0.12	3.1
L6	< 1000	524	442	581	2.71	0.06	2.2

Table 2 Intra-assay variation of the Fosun real-time PCR HBV DNA assay

No. of samples	Replicates	Mean of HBV DNA level ($\log_{10}$ copies/mL)	SD	CV (%)
3768	12	3.97	0.32	8.1
3785	12	6.00	0.12	2.0
3770	12	7.84	0.11	1.4

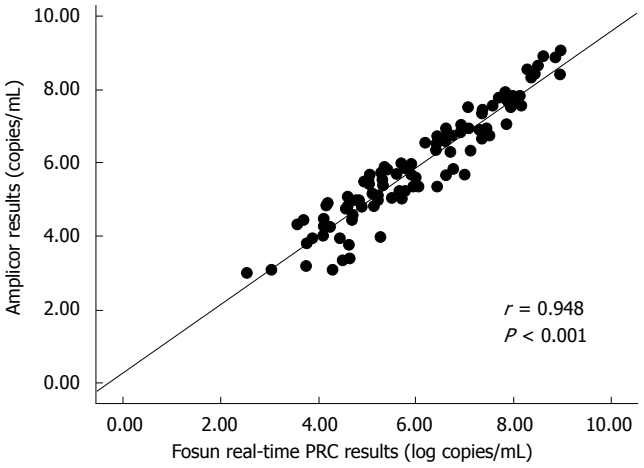

Figure 1 Correlation of HBV DNA levels measured by the Fosun real-time PCR and Amplicor tests.

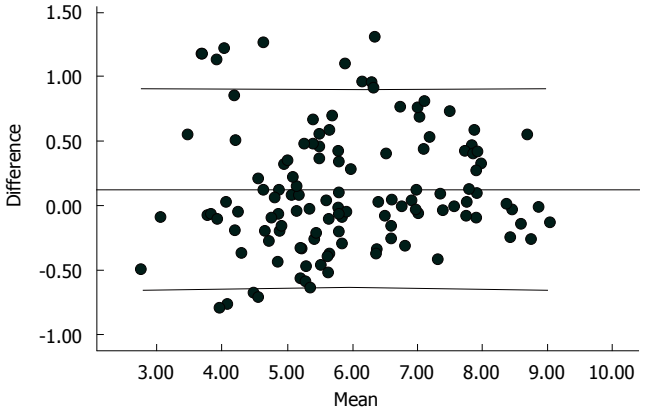

Figure 2 Differences between viral loads obtained by the two assays versus the average for each specimen.

parallel with the Fosun real-time PCR kits and the COBAS Amplicor HBV Monitor system. Samples were divided into three groups: group A, samples from healthy blood donors (n = 40); group B, samples from HBV infected patients with viral loads within the range of both tests (n = 43); group C, samples from HBV infected patients with viral loads above the upper limit of the Amplicor test (n = 75). These samples were diluted and re-tested with the Amplicor test. Viral loads were converted into logarithms. In group A, all the samples were negative for HBV with both assays. A comparison between the two assays was performed from samples in group B and C. Spearman's coefficient showed a significant correlation between the two assays (r = 0.948, P < 0.0001, Figure 1). The differences between viral loads obtained with the two assays versus the average for each specimen was analyzed with the Bland-Altman method[8] (Figure 2). Of the 158 samples, two (1.3%) samples had viral loads slightly above the upper limit and one (0.6%) sample was below the lower limit of the Fosun real-time PCR. However, viral loads of 75 (47.5%) samples were above the upper limit of the Amplicor test. In addition, HBV genotypes did not influence the quantitative results obtained with both assays.

Assay time and costs

The assay times and costs of both tests were compared. For a 24-sample work unit (controls were handled in the same way as clinical samples), Fosun real-time PCR required 3 h and the Amplicor test required 7 h. The cost of the Fosun real-time PCR per test, including consumables and DNA extraction reagents, was 5 US dollars. The cost of the Amplicor test was approximately 100 US dollars per test. If the samples were diluted and retested, the cost would be much higher.

DISCUSSION

China has the greatest burden of HBV infection in the world. Many commercially available HBV DNA assays in the United States and Europe are unaffordable for Chinese patients. Reliable but inexpensive HBV DNA assays are very important for control and treatment of HBV infection in low-income countries such as China. Fosun real-time PCR HBV DNA kits are produced by a Chinese company and approved by the Chinese SFDA for *in vitro* diagnostic use. Compared to the COBAS Amplicor test, this assay has equivalent reliability at a reduced cost.

Many studies have shown that real-time PCR is accurate, reproducible, sensitive, and rapid for quantitative detection of HBV levels in clinical samples[13,14,17-21]. Real-time PCR also has the widest dynamic range among all

the assays available commercially[14]. In this study, Fosun real-time PCR was able to quantitatively detect HBV in all seven reference sera from NICPBP, which showed a dynamic range of 8 logs. The majority (97.5%) of the HBV-positive samples used in this study were quantified within the manufacturer's recommended dynamic range for the Fosun real-time PCR, which indicates that this assay is superior to the Amplicor test for detection of HBV DNA in patients with high viral loads.

The results for the 158 clinical samples obtained with the Fosun real-time PCR and Amplicor tests were concordant very well. Comparison of the two assays for quantitative detection of HBV DNA levels in the 118 serum samples showed that the two sets of results were highly correlated ($r = 0.948$, $P < 0.001$). This was in accordance with the results of previous studies that have evaluated other real-time PCR assays for quantitation of HBV DNA[13,14,22-26].

One of the advantages of the Fosun real-time PCR kits is its low cost compared to that of the Amplicor test (5 *versus* 100 US dollars per test) and other commercially available tests[14]. With its low cost, it can be used to monitor antiviral therapy in economically disadvantaged patients with chronic HBV infection. As recommended by the American Association for Study of Liver Diseases and the Asian-Pacific Consensus Update Working Party on Chronic Hepatitis B, HBV DNA should be monitored at least every 3 mo during therapy. After the end of therapy, HBV DNA should be monitored monthly for the first 3 mo and then every 3-6 mo[27,28]. This will be a great economic burden for chronic hepatitis B patients in countries such as China, where most people have no medical insurance. Reliable and inexpensive assays for quantitation of HBV DNA are helpful for controlling HBV infection in such countries.

As with many other real-time PCR tests, the Fosun real-time PCR has no internal control included in the test. Internal controls can compensate for the differences in DNA extraction efficiency between specimens, and possible PCR inhibition in the reaction mixture[14,29-31]. In addition, standardization with international reference standards is also needed for wide acceptance of this assay. Further comparison with other commercially available assays such as the COBAS TaqMan 48 real-time PCR system will be helpful for evaluating the clinical performance of the Fosun real-time PCR assay.

In conclusion, the Fosun real-time PCR HBV DNA kit, with its much lower costs, is capable of providing reliable and rapid HBV DNA quantitation, which is useful for monitoring HBV DNA levels in patients with chronic hepatitis B.

COMMENTS

Background

Commercial real-time PCR assays are widely used for quantitation of hepatitis B virus (HBV) DNA in China. However, these assays have not been fully evaluated for their clinical performance.

Research frontier

Several methods are routinely used for quantitation of HBV DNA in serum or plasma samples. However, differences do exist among these methods. Comparison and evaluation of these methods are the focus of research efforts.

Related publications

Lindh *et al* and Gordillo *et al* have evaluated the clinical performance of commercial real-time PCR assays. However, they have not compared the cost and time between the real-time PCR and reference methods.

Innovations and breakthroughs

This study compared a real-time PCR assay widely used in China with the COBAS Amplicor test, which is widely used in the United States and Europe, for detection of HBV DNA in serum samples. Clinical performance, time required, and cost of the assays were analyzed.

Applications

This commercial PCR assay is suitable for quantitation of HBV DNA in serum samples.

Terminology

TaqMan PCR is one of the real-time PCR methods for the determination of copy number of PCR templates. It requires a pair of PCR primers and a fluorogenic probe, which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. The fluorescence can be monitored at frequent intervals during the PCR reaction with a real-time PCR machine.

Peer review

This is a well designed and prepared study with important conclusions. The most important point is that the real-time PCR is significantly cheaper than other commercial tests.

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CASE REPORT

Secondary aortoduodenal fistula

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Abstract

Aorto-duodenal fistulae (ADF) are the most frequent aorto-enteric fistulae (80%), presenting with upper gastrointestinal bleeding. We report the first case of a man with a secondary aorto-duodenal fistula presenting with a history of persistent occlusive syndrome. A 59-year old man who underwent an aortic-bi-femoral bypass 5 years ago, presented with dyspepsia and biliary vomiting. Computed tomography scan showed in the third duodenal segment the presence of inflammatory tissue with air bubbles between the duodenum and prosthesis, adherent to the duodenum. The patient was submitted to surgery, during which the prosthesis was detached from the duodenum, the intestine failed to close and a gastro-jejunal anastomosis was performed. The post-operative course was simple, secondary ADF was a complication (0.3%-2%) of aortic surgery. Mechanical erosion of the prosthetic material into the bowel was due to the lack of interposed retroperitoneal tissue or the excessive pulsation of redundantly placed grafts or septic procedures. The third or fourth duodenal segment was most frequently involved. Diagnosis of ADF was difficult. Surgical treatment is always recommended by explorative laparotomy. ADF must be suspected whenever a patient with aortic prosthesis has digestive bleeding or unexplained obstructive syndrome. Rarely the clinical picture of ADF is subtle presenting as an obstructive syndrome and in these cases the principal goal is to effectively relieve the mechanical bowel obstruction.

Key words: Aorto-duodenal fistula; Surgery; Dyspepsia; Duodenotomy; Explorative laparotomy

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INTRODUCTION

Aorto-duodenal fistulae (ADF) are the most frequent aorto-enteric fistulae (80%). The most frequent presenting sign of ADF is upper gastrointestinal bleeding (UGI). We report the first case of a man with a secondary aortoduodenal fistula presenting with a history of persistent occlusive syndrome, which was treated with surgical exploration.

CASE REPORT

A 59-year old male patient who underwent an aortic-bi-femoral bypass 5 years ago, was admitted to the Emergency Room for a 5-day history of persistent occlusive syndrome with dyspepsia and biliary vomiting. Physical examination revealed a distended abdominal pain. Full blood examination was unremarkable.

Computed tomography (CT) scan showed in the third duodenal segment the presence of an area with the characteristics of inflammatory tissue including air bubbles between the duodenum and aortic-bi-femoral prosthesis adherent to the third duodenal portion ("comma sign") (Figure 1). Microbiological cultures and scintigraphy were unremarkable. Esophago-gastro-duodenoscopy showed the aortic prosthesis crossing the third segment of the duodenal wall occluding the intestinal lumen (Figure 2). At laparotomy, after viscerolisis, the prosthesis was detached from the duodenal wall and the intestine failed to close transversely (Figure 3). To protect the intestinal wall, a pedunculated fragment of the greater omentum was placed between the duodenum and aortic bypass. Furthermore, a gastrojejunal Roux anastomosis was employed. The prosthesis was not changed because there were no local or systemic signs of infection. The post-operative course was uneventful. The patient was discharged 10 days after operation and received a regular follow-up for 6 mo.

DISCUSSION

ADF are the most frequent aorto-enteric fistulae (80%)[1]. They may be primary due to a spontaneous communication between the lumen of aortic aneurysm

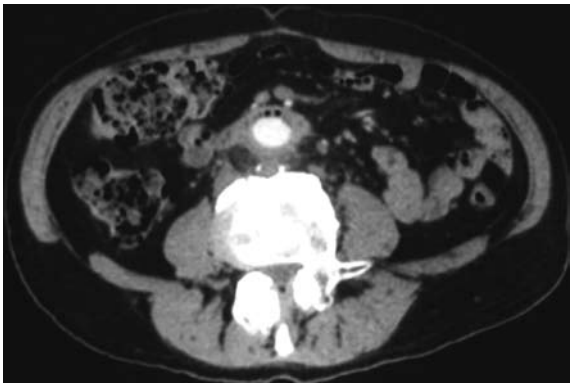

Figure 1 CT scan showing an area with the characteristics of inflammatory tissue including air bubbles between the duodenum and aortic-bi-femoral prosthesis adherent to the third duodenal portion.

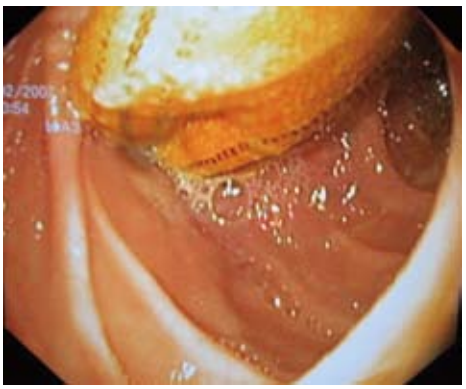

Figure 2 Esophago-gastro-duodenoscopy showing the aortic prosthesis crossing the third segment of the duodenal wall occluding the intestinal lumen.

and intestinal loop, or secondary due to surgical repair of aneurysms with prosthetic implants. The most frequent presenting sign of ADF is UGI bleeding. Clinical suspicion is essential in the diagnosis of ADF and the most commonly used techniques for its diagnosis are esophago-gastro-duodenoscopy (EGDS) and CT.

Secondary ADF is an uncommon (0.3%-2%) and life-threatening long-term complication of aortic reconstructive surgery[2]. Although the exact pathogenesis remains speculative, mechanical erosion of the prosthetic material into the adjacent bowel may be due to the lack of interposed retroperitoneal tissue or the excessive pulsation of redundantly placed grafts or septic procedures (low-grade, Staphylococcus epidermidis "biofilm" infection). Finally, some authors have described cases of inadequate prosthetic materials[2,3]. In our case, ADF formation was related to graft pulsation on the duodenal wall (in this patient, the blood pressure was stably high and not controlled by drugs).

The time between the first intervention and development of fistula can vary from months to years[3,4]. The presentation is often subtle with herald bleeding followed by a period of grace, or catastrophic bleeding, or rarely an episode of intestinal obstruction. The third or fourth duodenal segment is the most frequently involved site. In Dacron prosthesis patients, fistula develops in the

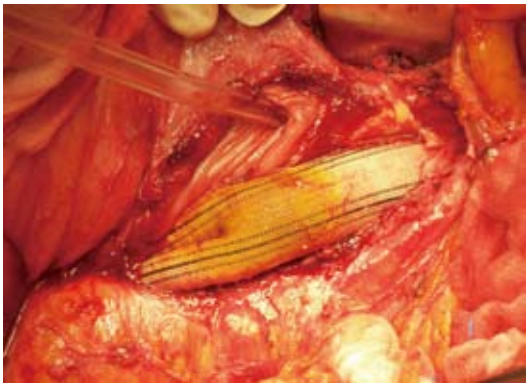

Figure 3 Isolated prosthesis in the context of eroded duodenal wall during laparotomy (the tube indicates the proximal duodenal lumen).

proximal graft tract opening in the third segment of the duodenum[1]. The right diagnosis of ADF can be difficult. It needs an early and correct suspicion index to establish the diagnosis of ADF in all patients who have undergone aortic graft surgery and present with gastrointestinal haemorrhage, sometimes fever, abdominal pain or mass or obstructive syndrome[5,6].

Though difficult, diagnosis can be achieved through EGDS or CT scan. Additional diagnostic procedures are often not useful. EGDS, the main diagnostic procedure, is able to demonstrate the fistula and rule out other possible causes of obstruction, but the most important tool to achieve diagnosis is clinical suspicion[3,6]. Because of the high mortality and morbidity, associated with secondary aorto-enteric fistula, surgical treatment is always recommended. Explorative laparotomy is the treatment of choice. Surgical procedures help guide the diagnosis and constitute the main part of treatment with suture of the duodenum and evaluation of vascular prosthesis.

In the case of non-treated aortic-enteric fistula presenting with massive UGI-bleeding, the mortality rate is near to 100%. While the morbidity (limb loss in 10%-40%) and mortality related to treated ADF are also high (75%) and require preventive measures, including more particularly delicate surgery and antibiotic therapy in case of any episode of infection. Several surgical procedures are possible[4,7].

ADF must be suspected whenever a patient with aortic prosthesis has digestive bleeding or unexplained obstructive syndrome[8]. More rarely, the clinical picture of ADF is subtle presenting as an obstructive syndrome and in these cases the principal goal is to effectively relieve the mechanical bowel obstruction. Further study is necessary to establish the more effective diagnostic mode.

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Natural killer T cells and non-alcoholic fatty liver disease: Fat chews on the immune system

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Abstract

Natural killer T cells (NKT) are an important subset of T lymphocytes. They are unique in their ability to produce both T helper 1 and T helper 2 associated cytokines, thus being capable of steering the immune system into either inflammation or tolerance. Disruption of NKT cell numbers or function results in severe deficits in immune surveillance against pathogens and tumor cells. Growing experimental evidence suggests that hepatosteatosis may reduce resident hepatic as well as peripheral NKT cells. Those models of hepatosteatosis and the change in NKT cell numbers are associated with a disruption of cytokine homeostasis, resulting in a more pronounced release of proinflammatory cytokines which renders the steatotic liver highly susceptible to secondary insults. In this letter to the editor, we focus on recently published data in the *World Journal of Gastroenterology* by Xu and colleagues demonstrating reduced peripheral NKT cells in patients with non-alcoholic fatty liver disease, compare those findings with ours and others in different animal models of hepatosteatosis, and hypothesize about the potential underlying mechanism.

Key words: Obesity; Hepatosteatosis; Metabolic syndrome

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TO THE EDITOR

We are excited to read a recent report in the *World Journal of Gastroenterology* by Xu and colleagues demonstrating a reduction in the numbers of peripheral natural killer T cell (NKT) cells in patients with non-alcoholic fatty liver disease[1]. A growing body of experimental data demonstrates a link between hepatosteatosis and defects in NKT numbers and/or function. Over the past years, reductions in NKT cell numbers was reported in the fatty liver by a number of groups including ours[2-4]. Observations by our group show an inverse correlation between hepatic NKT cell numbers with the accumulation of hepatic lipid using the choline-deficient diet model of hepatosteatosis[4]. Although different animal models of diet-induced hepatic steatosis using high fat, high sucrose and choline-deficient diet or leptin deficient *ob/ob* mice showed depletion or decrease in hepatic NKT cell numbers[2-4], it has remained unclear if those findings were relevant to human fatty liver disease.

NKT cells play a critical role both in the liver and peripherally in the regulation of the innate and adaptive immune response. Decreased hepatic NKT cell numbers correlate with increased local production of pro-inflammatory T helper 1-associated cytokines[4,5]. We and others have shown that T helper 1-associated cytokines, such as tumor necrosis factor alpha, interleukin 12 and interferon gamma, are significantly elevated in steatotic livers in mouse models of obesity[3-5]. Similar findings have been reported in obese individuals with low levels of adiponectin and increased levels of circulating tumor necrosis factor alpha[6,7], although those studies did not examine hepatic or peripheral NKT cell numbers. NKT cells are unique in their ability to produce both T helper 1- and T helper 2-associated cytokines. The loss of NKT cells likely contributes to disrupted cytokine balance within the liver[5,8,9]. Consistent with their function as a regulator of the immune response, loss of NKT cell-associated interleukin 4 production promotes beta cell destruction in the *db/db* mouse model of type I diabetes[10]. Finally, NKT cells also function to clear tumor cells within the liver, as NKT cell activation promotes tumor cell clearance in several mouse models of hepatic metastasis[11]. Therefore, it is apparent that NKT cells play an important immunological and potentially regulatory role in both the liver and peripheral organs. The ability of hepatic lipid accumulation to alter this multifunctional cell population may have important and widespread pathophysiological consequences.

It remains unclear how hepatosteatosis reduces hepatic

and peripheral NKT cells. Previous studies implicated that interleukin 12 is a key activator of NKT cells, a process which is linked to activation-induced cell death of this cell population[12]. Therefore, it is likely that increased levels of interleukin 12 in fatty livers directly reduce the viability of NKT cells. We hypothesize that increased interleukin 12 expression in combination with hepatic lipid accumulation might be directly responsible for the reduced NKT cell population in non-alcoholic fatty liver disease (NAFLD).

The current study by Xu and colleagues is the first of its kind to establish this inverse relationship between NKT cell numbers and hepatosteatosis in humans and provides strong support to animal models of steatotic liver disease and immune cell dysfunction. Future research should focus on hepatic resident NKT cell numbers in humans suffering from NAFLD, and the relationship between hepatic and systemic cytokine levels of those patients should be monitored.

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