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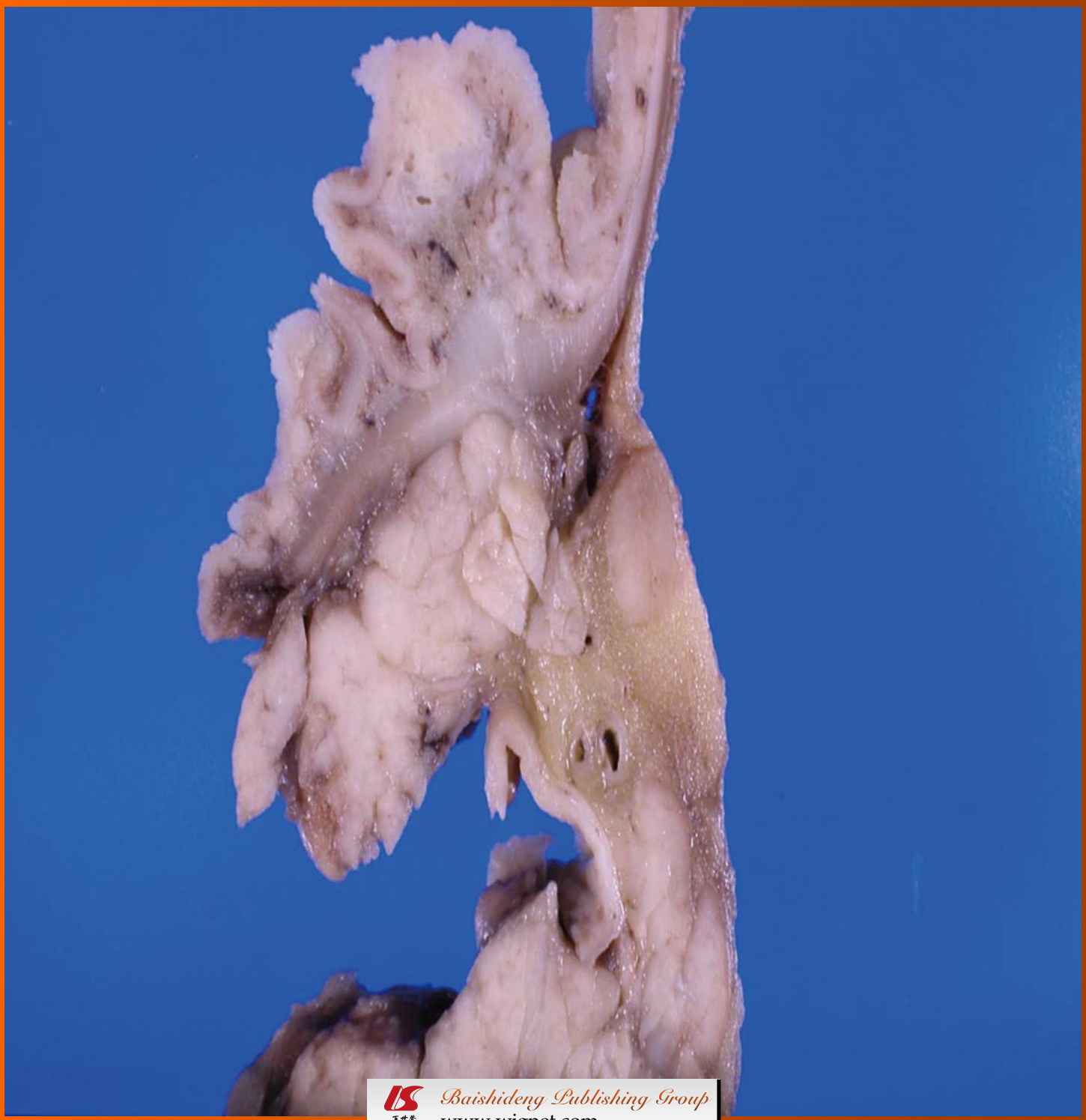

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EDITORIAL

Adenomyoma of the small intestine

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Abstract

Adenomyoma of the gastrointestinal tract is a rare benign tumor-like lesion. The small intestine is the second most frequent location, usually in the periampullary area, but the lesion also occurs in the jejunum and ileum. While adenomyoma of the Vaterian system is primarily diagnosed in adults, more than half of reported cases of jejunal and ileal adenomyoma have been diagnosed in pediatric patients. Adenomyoma of the periampullary area usually presents with biliary obstruction or abdominal pain, whereas jejunal and ileal adenomyoma usually presents with intussusception or is incidentally discovered during surgery or autopsy. Since endoscopic and radiological examination yields uncharacteristic findings, histopathological evaluation is important in adenomyoma diagnosis. Pathologically, adenomyoma consists of glandular structures of various sizes and interlacing smooth muscle bundles that surround the glandular elements. The pathogenesis of adenomyoma is generally considered to be either a form of hamartoma or a pancreatic heterotopia. Although limited resection is considered the most effective treatment, pancreaticoduodenectomy is often performed when the lesion occurs in the periampullary area due to preoperative misdiagnosis as a carcinoma. It is, therefore, important that clinicians and pathologists maintain current knowledge of the disease to avoid inaccurate diagnosis, which could lead to unnecessary surgery.

Key words: Adenomyoma; Small intestine; Biliary obstruction; Intussusception; Hamartoma; Heterotopic pancreas

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INTRODUCTION

Adenomyoma of the gastrointestinal (GI) tract, also referred to as a myoepithelial hamartoma, adenomyomatous hamartoma or foregut choristoma, is a benign tumor-like lesion histologically characterized by glandular structures lined by cuboidal to tall columnar epithelium and surrounded by bundles of smooth muscle. It occurs mainly in the pyloric region of the stomach[1]. The small intestine is the second most frequent location, usually in the periampullary area, but it also occurs in the jejunum and ileum. The lesion is very rare and there have been only a few reports of case series of periampullary adenomyoma[2,3]. Most cases of jejunal and ileal adenomyoma have been reported as single case reports and, to the best of our knowledge, there have been only 26 reported cases[4-26].

Although the pathogenesis of adenomyoma remains unclear, it is hypothesized to be either a form of hamartoma or an incomplete heterotopic pancreas. As endoscopic and radiological examination yields uncharacteristic findings, histopathological evaluation is important in adenomyoma diagnosis. It is important that clinicians and pathologists maintain current knowledge of the disease

to avoid making inaccurate diagnoses that may lead to unnecessary surgery. To aid in the acquisition of this important knowledge, we review the clinical and pathological features of adenomyoma of the small intestine and discuss its pathogenesis.

CLINICAL FEATURES

Epidemiology

As mentioned above, adenomyoma of the small intestine is rare, especially that occurring in the small intestine distal to the duodenum. The actual incidence is unclear because very few cases have been reported. A further complicating factor in determining its true incidence is that its reportedly low incidence may be partly attributed to underreporting or nonrecognition of the condition by both surgeons and pathologists[17]. We have diagnosed 3 cases of asymptomatic adenomyoma of the jejunum or ileum at autopsy in our institution within the past 8 years; this fact suggests that its incidence is higher than suspected.

In the investigation of 13 cases of adenomyoma of the Vaterian system treated by extensive surgery, the patient age ranged from 38 to 78 years (mean 63 years) and the male-to-female ratio was 6:7[2]. On the other hand, there have been only 26 reported cases of jejunal and ileal adenomyoma[4-26] and the patient age ranges from 2 d to 82 years (mean 25 years), including 15 pediatric patients and 11 adult patients. The male-to-female ratio is approximately 2:1. The lesion occurs 2 to 3 times more frequently in the ileum than in the jejunum. One lesion was found in a Meckel diverticulum[17].

Symptoms and signs

Symptoms of adenomyoma of the GI tract depend on the location of the lesion and patient age. Adenomyoma of the periampullary area usually presents with biliary obstruction (obstructive jaundice) or abdominal pain, symptoms that recur after sphincterotomy[2]. Several cases have been incidentally detected during systemic examination for other diseases. One reported case presented with acute recurrent pancreatitis[27].

Jejunal and ileal adenomyoma of pediatric patients usually presents with intussusception, but 1 reported case presented with intestinal obstruction[6]. In adult patients, intussusception is an infrequent complication, with many reported cases having been incidentally detected during surgery for other diseases or during autopsy. Several cases have presented with GI bleeding (melena)[15,25].

Endoscopy

On endoscopic examination, adenomyoma of the duodenum is detected as a submucosal tumor-like nodule covered by normal mucosa. Although it is generally difficult to detect jejunal or ileal lesion by endoscopy, 1 reported case of adenomyoma of the proximal jejunum was identified by push enteroscopy[25].

Radiographic findings

Adenomyoma of the GI tract may be detected as an enhancing polypoid lesion by abdominal computed tomography (CT)[21]. Periampullary adenomyoma may be detected as an abnormal shadow on endoscopic retrograde cholangiopancreatography (ERCP)[28]. When ampullary adenomyoma causes stenosis or obstruction of the biliary tract, bile duct dilatation can be detected by abdominal ultrasonography, abdominal CT, ERCP and magnetic resonance cholangiopancreatography; bile duct obstruction can be confirmed by percutaneous transhepatic cholangiography.

PATHOLOGICAL FEATURES

Gross appearance

Grossly, adenomyoma of the GI tract is an intramural nodule covered by mucosa and it protrudes into the lumen (Figure 1A and B). The diameter of adenomyoma in reported cases ranges from 0.6 cm to 4.5 cm.

Microscopic findings

Histologically, adenomyoma of the small intestine mainly occupies the submucosa (Figure 2) and often extends into the muscularis propria. The lesion consists of glandular structures of various sizes and interlacing smooth muscle bundles surrounding the glandular elements (Figure 3). Cystically dilated glands are usually observed. The glandular structures are lined by cuboidal to tall columnar epithelium with basally oriented nuclei. Goblet cells are occasionally interspersed (Figure 4A) and we previously reported a case in which Paneth cells were also observed (Figure 4B)[23]. Those glands are surrounded by interlacing smooth muscle bundles. Myofibroblasts and fibroblasts may also proliferate[2,3]. Both the epithelial and smooth muscle cells lack nuclear atypia. Pancreatic acini and islet tissue are not present. Pathological diagnosis by biopsy specimen is usually difficult, partly because the lesion mainly occupies the submucosa.

Immunohistochemical staining

Immunohistochemically, the glandular element of adenomyoma of the small intestine is positive for cytokeratin (CK) 7 (Figure 5A) and negative for CK 20 (Figure 5B)[23,26], while normal intestinal epithelial cells around the lesion are negative for CK 7 and positive for CK 20. The glandular epithelial cells of the lesion do not express CDX-2, a marker of intestinal mucosal epithelium[26]. The smooth muscle cells surrounding the glandular elements are positive for α-smooth muscle actin and desmin (Figure 6)[20,23].

Differential diagnosis

Differential diagnoses of adenomyoma of the small intestine include enteritis cystica profunda, pneumatosis cystoides intestinalis, adenocarcinoma and hamartomatous polyp in Peutz-Jeghers syndrome. Cysts of enteritis

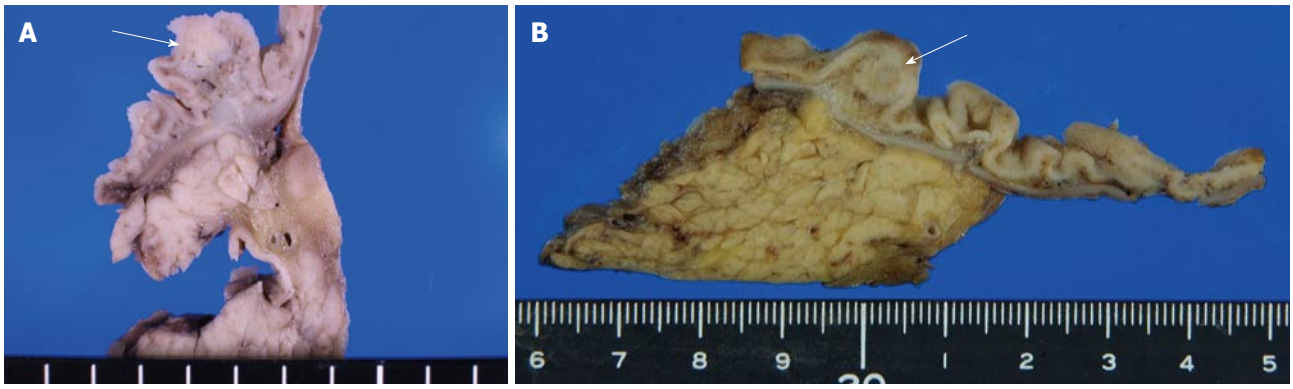

Figure 1 Gross appearance of adenomyoma of the periampullary region. The lesions are observed as intramural nodules covered by mucosa and they protrude into the lumen (A, B, arrows).

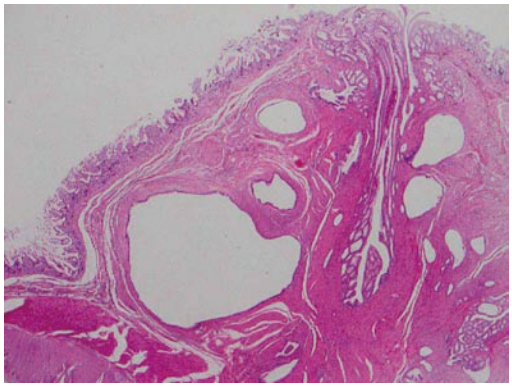

Figure 2 Low-power view of adenomyoma of the small intestine. A nodular lesion mainly occupies the submucosa (hematoxylin and eosin stain, × 10).

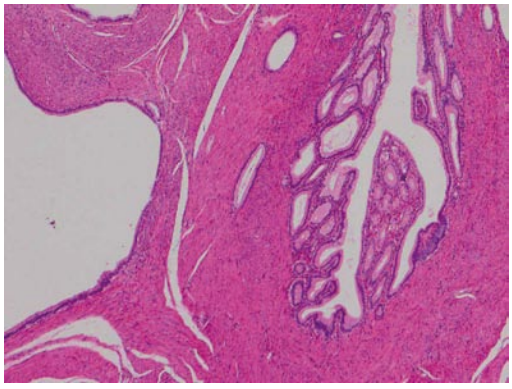

Figure 3 High-power view of adenomyoma of the small intestine. The lesion consists of glandular structures of various sizes and interlacing smooth muscle bundles (hematoxylin and eosin stain, × 40).

cystica profunda are not surrounded by smooth muscle bundles. In pneumatosis cystoides intestinalis, the cysts contain gas and are lined by multinucleated giant cells, while the glands and cysts of the adenomyoma are lined by epithelial cells. The characteristics of adenomyoma that differentiate it from adenocarcinoma include the absence of cellular atypia and desmoplastic stroma and the presence of smooth muscle bundles surrounding the glands and cysts. In Peutz-Jeghers syndrome, the essential feature is branching cores of muscular fibers derived from the muscularis mucosae and covered by normal mucosa, while adenomyoma is located in submucosa and/or muscularis propria.

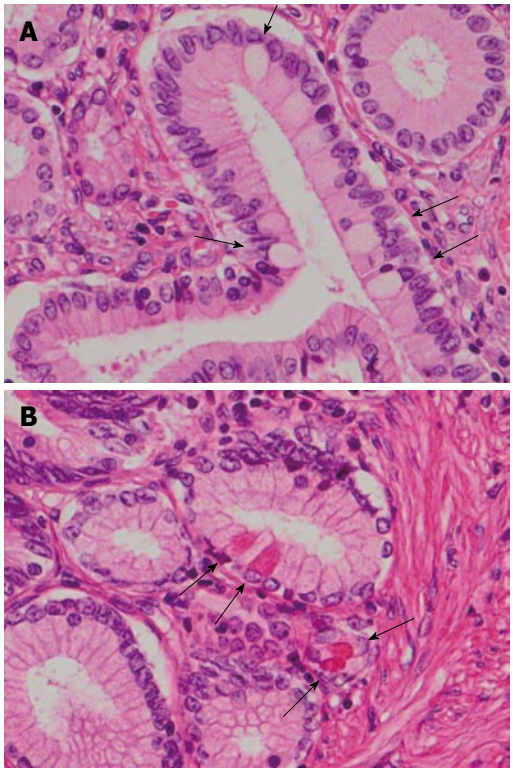

Figure 4 Appearance of goblet cells (A, arrows) and Paneth cells (B, arrows) in the intestinal adenomyoma (hematoxylin and eosin stain, × 400).

TREATMENT AND PROGNOSIS

Endoscopic or surgical limited resection of the lesion is considered the most effective treatment for adenomyoma of the periampullary region. However, pancreaticoduodenectomy is often performed because the lesion is frequently preoperatively misdiagnosed as a carcinoma. Intraoperative frozen section diagnosis is useful to avoid excessive surgery.

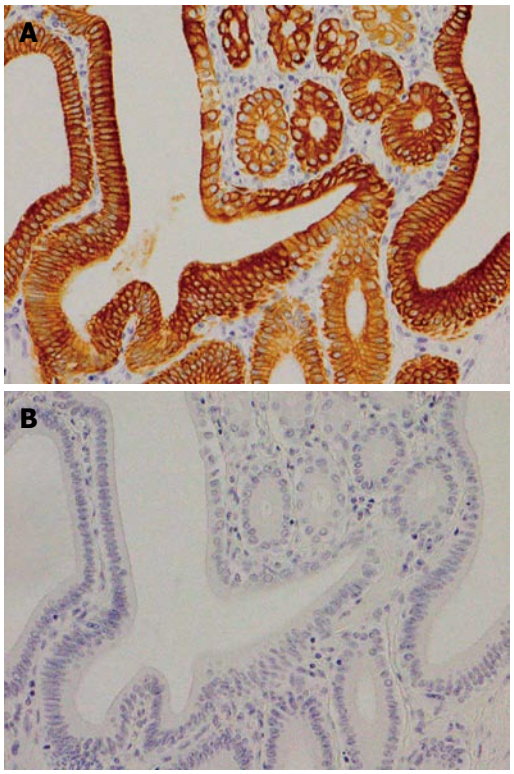

Figure 5 Results of immunohistochemical staining for cytokeratin 7 and cytokeratin 20. The glandular element of the lesion is positive for cytokeratin 7 (A) and negative for cytokeratin 20 (B) (× 200).

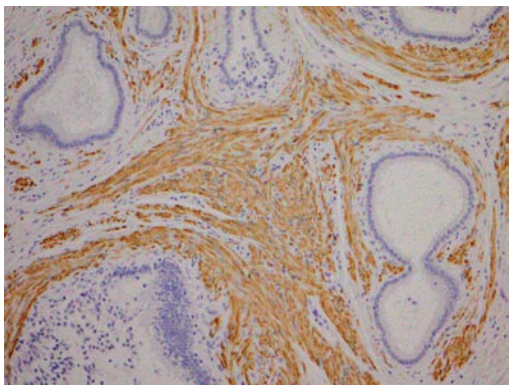

Figure 6 Results of immunohistochemical staining for desmin. Smooth muscle cells surrounding the glandular elements are positive for desmin (× 100).

A partial enterectomy or simple resection of the lesion is performed for a jejunal or ileal adenomyoma complicated by intussusception. When it is not complicated by intussusception, simple resection of the lesion is sufficient treatment. Intraoperative frozen section diagnosis is also useful when the lesion exists in this location. As adenomyoma of the GI tract is a benign lesion, the prognosis for its treatment is very good.

Follow-up is considered to be a potential option for avoiding unnecessary surgery for benign adenomyoma, particularly in the jejunum and ileum, if the size of the lesion is small. Follow-up study may elucidate the natural course of the lesion.

PATHOGENESIS

As mentioned above, the pathogenesis of adenomyoma of the GI tract is generally considered either a form of a hamartoma or a pancreatic heterotopia, although this is not fully understood. The term "hamartoma" refers to an excessive but focal overgrowth of cells or tissues native to the organ in which it occurs, while the term "heterotopia" refers to a growth of microscopically normal cells or tissues in an abnormal location.

Gal *et al*[9] reported 3 cases of adenomyoma of the small intestine and suggested that "adenomyomas should be regarded as hamartomas of the GI tract" based on the fact that those cases contained goblet cells, argentaffin cells and smooth muscle stroma. Several authors reported cases with the transitional area between the epithelial component of the adenomyoma and epithelium of the overlying mucosa and considered it to be evidence that the epithelial component of the lesion originated from the epithelium of the small intestine[21,26].

In general, CK 7 is distributed in the pancreatic duct epithelium but is essentially absent in GI epithelium. On the other hand, CK 20 is distributed in the GI epithelium but is absent in pancreatic duct epithelium[29]. Accordingly, the pattern of cytokeratin expression [CK 7 (+), CK 20 (-)] of the glandular element of adenomyoma coincides with that of the pancreatic duct epithelium but not with that of the intestinal epithelium, thus supporting the heterotopic pancreas theory. This theory was further supported by Babál *et al*[30]'s detection of histochemical reactivities of the duodenal adenomyoma similar to the reactivities of duct epithelium in the neighboring pancreas, as well as Yao *et al*[17]'s reporting of a case of adenomyoma occurring in a Meckel diverticulum. In their examination of a case series of adenomyoma of the Vaterian system, Handra-Luca *et al*[2] identified that 3 of 13 cases were characterized by pancreatic heterotopias with both exocrine and endocrine pancreatic tissue being present in continuity with the adenomyoma.

In our opinion, the heterotopic pancreas theory regarding the lesion's pathogenesis might be more convincing than the hamartoma theory. The appearance of goblet cells and argentaffin cells might be explained by a metaplastic mechanism, while the presence of hyperplastic smooth muscle tissue might be explained by secondary muscle proliferation caused by a stimulus emanating from misplaced epithelium. We acknowledge that whether transition between the epithelial component of the lesion and the epithelium of the overlying mucosa is truly evidence of hamartomatous pathogenesis remains open to question. However, of course, further examinations are necessary to determine the pathogenesis of the lesion.

CONCLUSION

Adenomyoma of the GI tract is a rare benign tumor-like lesion whose pathogenesis remains not fully understood. When adenomyoma occurs in the Vaterian system, its

clinical differentiation from a carcinoma is difficult, often leading to a needless pancreaticoduodenectomy. To avoid unnecessary radical surgery, clinicians and pathologists should maintain current knowledge of the lesion and the most effective means of treatment.

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EDITORIAL

Treatment strategy for gastric non-invasive intraepithelial neoplasia diagnosed by endoscopic biopsy

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Abstract

Treatment strategies, whether as follow-up or "total incisional biopsy" for gastric noninvasive intraepithelial neoplasia diagnosed by examination of an endoscopic forceps biopsy specimen, are controversial due to problems associated with the diagnostic accuracy of endoscopic forceps biopsy and questions about the safety and efficacy of endoscopic treatment. Based on the histological findings of the biopsy specimen, it is difficult to differentiate between reactive or regenerative changes, inflammation and neoplastic changes, intraepithelial and invasive tumors. Therefore, gastric neoplasia diagnosed as noninvasive intraepithelial often develop into invasive carcinoma during follow-up. Recent advances in endoscopic modalities and treatment devices, such as image-enhanced endoscopy and high-frequency generators, may make endoscopic treatment, such as endoscopic submucosal dissection (ESD), a therapeutic option for gastric intraepithelial neoplasia, including low-grade neoplasms. Future studies are required to evaluate whether ESD is a valid strategy for gastric intraepithelial neoplasm with regard to safety and cost effectiveness.

Key words: Gastric intraepithelial neoplasia; Adenoma; Dysplasia; Endoscopic submucosal dissection; Endoscopic mucosal resection; Endoscopic resection; Adenocarcinoma

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INTRODUCTION

Gastric cancer is the second most common cause of death from cancer worldwide[1,2] and more than half of the world's gastric cancer cases arise in eastern Asia. Early gastric cancer (EGC) is typically small, asymptomatic and has a good prognosis[3,4], but advanced gastric cancer has higher mortality rate[5]. Therefore, early detection and treatment is important for reducing the gastric cancer mortality rate. In particular, early detection of EGC is important to improve the prognosis of patients with gastric cancer. Surveillance

with endoscopy and biopsy sampling is important in patients with premalignant lesions and may lead to the early detection of cancer[6].

Gastric intraepithelial dysplasia/adenomas are considered to be precancerous lesions with a variable clinical course[7,8]. The term intraepithelial dysplasia/adenoma, however, is complex and confusing because of the lack of a uniform classification regarding the features that differentiate between dysplasia/adenoma and EGC. Moreover, it is difficult to differentiate gastric epithelial dysplasia/adenoma and ECG using biopsy specimens because of the inaccuracy of obtaining a biopsy specimen from a malignant region of cancer in an adenoma. This diagnostic inconsistency leads to inappropriate treatment and often results in over- or under-treatment of gastric intraepithelial neoplasias.

In this editorial, we discuss clinical problems in making a diagnosis and treating gastric intraepithelial neoplasia lesions as premalignant.

CHANGES IN CLASSIFICATION FOR GASTRIC INTRAEPITHELIAL NEOPLASIA

In the early 1980s, guidelines for the diagnosis and grading of gastric epithelial neoplasia were developed and a three-stage classification (mild, moderate and severe dysplasia) was proposed. The term "dys" means abnormal and "plasia" means growth; thus, dysplasia is the term for abnormal growth of epithelial cells. Dysplasia is generally defined as unequivocally neoplastic epithelium that may be associated with or develop into invasive adenocarcinoma[9-11]. On the other hand, lesions that most European and American pathologists identify as dysplasia are often considered adenocarcinoma in Japan because, according to the Japanese viewpoint, gastric carcinoma is diagnosed based on nuclear and structural atypia, even when invasion is absent. Therefore, the reports of many Japanese and western pathologists show considerable differences. Schlemper *et al*[12,13], however, reported that diagnoses based on nuclear and structural atypia are somewhat discrepant between biopsy and resection specimens. They concluded that this may be the reason for the relatively high incidence and good prognosis of gastric carcinoma in Japan compared to western countries. In addition, the term adenoma is applied mostly to macroscopically protruding or superficially elevated lesions in Europe and America but in Japan the term applies to all gross types of the lesions: flat, elevated and depressed.

This confusion has led to several classifications for the terminology between non-neoplastic changes and early invasive cancer[9,11,14-16]. In September 1998, approximately 30 pathologists from 12 countries met in Vienna just before the World Congress of Gastroenterology and reached a consensus on the terminology for gastrointestinal epithelial neoplasia, termed the Vienna classification[17]. In this classification, "high-grade adenoma/dysplasia", "non-invasive carcinoma (carcinoma *in situ*)" and "suspected invasive carcinoma" were clustered into a single category (category 4), termed "noninvasive high-grade neoplasia" to eliminate the diagnostic discrepancies between western and Japanese pathologists. Because these three diagnoses cannot be reproducibly distinguished and the treatment recommendation would be the same for each diagnosis, these lesions are considered to be premalignant lesions[18-20]. At the beginning of 2000, the Vienna classification was revised[21] and, for a similar reason, intramucosal carcinoma was added as a fourth subcategory of category 4, because it is often hard to determine whether there is invasion into the lamina propria and because from a therapeutic viewpoint, the distinction between any of the four subcategories is irrelevant. After the revised Vienna classification was introduced, agreement on the diagnosis improved to 80% for gastric lesions[22]. The practical difficulty for diagnosing gastric epithelial dysplasia, however, remains the interpretation by clinicians of the terminology used by pathologists. The use of the term dysplasia confuses clinicians because endoscopy and surgery are linked to legal and social problems and most western surgeons will not operate if pathologists do not clearly diagnose the dysplasia as cancer. Therefore, it is currently not feasible to eliminate the diagnoses of dysplasia in the gastric mucosa. The recently revised new World Health Organization classification of neoplasia of the gastrointestinal tract was published in 2010, in which the term dysplasia is described as "intraepithelial neoplasia (dysplasia)" with dysplasia in parentheses.

Description of intraepithelial neoplasia

Endoscopy is the most sensitive and specific diagnostic tool for gastric neoplasms[23]. It is possible to detect slight changes in color and architecture of the mucosal surface that suggest EGC, in particular, using high-resolution endoscopy[24] and narrow band imaging[25] with chromo endoscopy[26] such as indigo carmine solution. Not to miss a lesion of gastric intraepithelial neoplasia, we often use biopsy specimens as a golden standard for diagnosis. In some cases, we supplement by ultrasonographical assessments of the depth of invasion to judge the lesions that suggest EGC. Those biopsy specimens are diagnosed as below (Table 1).

Indefinite for intraepithelial neoplasia - category 2 in the Vienna classification: Depending on the condition of a biopsy sample, particularly small biopsy specimens, it is occasionally difficult to distinguish whether a lesion is neoplastic or non-neoplastic, reactive or regenerative. A diagnosis of "indefinite for dysplasia" is not a strict biological entity but rather a temporary term that is necessary to keep the patient in follow-up and to obtain more biopsies to make a definitive diagnosis.

Low-grade intraepithelial neoplasia - category 3 in the Vienna classification: Low-grade intraepithelial neoplasia (LGIN) belongs to this category. This lesion shows a slightly modified mucosal architecture, includ-

Table 1 Definition of intraepithelial neoplasia

	Definition	
	Japanese view	Western view
Indefinite for intraepithelial neoplasia	A temporary term It is difficult to distinguish whether a lesion is neoplastic or non-neoplastic, or reactive or regenerative	A temporary term
LGIN	Characterized by a slightly modified mucosal architecture, including the presence of tubular structures with budding and branching, papillary enfolding, crypt lengthening with serration and cystic changes	
HGIN	Characterized by an increasing architectural distortion with glandular crowding and prominent cellular atypia without stromal invasion	
Adenocarcinoma/carcinoma	Diagnosed on nuclear and structural atypia, even when invasion is absent[45]	Diagnosed when evident invasive growth of neoplastic epithelium into the lamina propria of the mucosa or beyond is observed[46]

LGIN: Low-grade intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia.

Table 2 Histological discrepancy rates between biopsy and endoscopic resection sample *n* (%)

Reports (yr)	Endoscopic biopsy	Resected specimens		Overall
		Underdiagnosis[1]	Overdiagnosis[2]	Discrepancy[3]
Yoon *et al*[47], 2006	Tubular adenoma	2/41 (4.9)	2/41 (4.9)	4/41 (9.8)
Jung *et al*[29], 2008	LGIN	31/74 (42)	-	-
	HGIN	36/40 (90)	2/40 (5)	38/40 (95)
Lee *et al*[48], 2010	IN	114/311 (37)	41/311 (13)	155/311 (50)
	Carcinoma	7/86 (8.1)	16/86 (19)	23/86 (26)
	Total	121/397 (30)	57/397 (14)	178/397 (45)
Kato *et al*[27], 2010	IN	255/468 (44)	4/468 (1.7)	259/468 (46)

[1]Underdiagnosis was defined as if endoscopic biopsy showed tubular adenoma/ intraepithelial neoplasia but resected specimens finally led to the diagnosis of adenocarcinoma/carcinoma; [2]Overdiagnosis was defined as if endoscopic biopsy showed tubular adenoma/intraepithelial neoplasia or adenocarcinoma/carcinoma but resected specimens finally led to the diagnosis of non-neoplastic, reactive, regenerative or tubular adenoma, respectively; [3]Discrepancy was defined as if endoscopic biopsy does not correspond with resected specimen. This can be calculated using resected specimen as a golden standard. LGIN: Low-grade intraepithelial neoplasia; IN: Intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia.

ing the presence of tubular structures with budding and branching, papillary enfolding, crypt lengthening with serration and cystic changes.

High-grade intraepithelial neoplasia - category 4.1 in the Vienna classification: There is increasing architectural distortion with glandular crowding and prominent cellular atypia. Tubules can be irregular in shape, with frequent branching, budding and intra-luminal bridges, but there is no stromal invasion. The pleomorphic nuclei show prominent amphophilic nucleoli and a loss of polarity. Increased proliferative activity is present throughout the epithelium.

Difficulty of accurate diagnosis based on biopsy

Endoscopic forceps biopsy is the gold standard for histological diagnosis of gastric epithelial neoplasia. A pathological diagnosis established by biopsy specimen, however, sometimes results in an "under-diagnosis" when compared with diagnosis established by resected specimens. The frequency of discrepant diagnoses between biopsy specimens and the corresponding resected specimens of the same lesions ranges widely in published reports (Table 2). At least, high-grade intraepithelial neoplasia (HGIN) may already have a high probability of a carcinoma. We recently reported that the under-diagnosis rate of intraepithelial neoplasia proven by biopsy was 44% (95% CI: 39%-49%). Moreover, in that study, there were 2 lesions (0.42%) of adenocarcinoma with submucosal invasion of more than 500 μm, one of which involved the lymphatic system[27]. The reasons for the difficulty in making an accurate diagnosis based on a biopsy specimen are as follows: (1)the structural atypia of both adenoma and well-differentiated adenocarcinoma is too subtle to detect in small biopsy specimens; (2) cancer sometimes exists focally in the lesion and a sampling error might occur; and (3) regeneration of atypia induced by gastritis induces histological modification (Figure 1). Recent reports tend to show a higher under-diagnosis rate between biopsy and endoscopic resection samples than previous studies. Moreover, active inflammation of the gastric mucosa infected by *Helicobacter pylori* may conceal neoplastic architectural distortion and lead to false negative results. We speculate that recent optical advances in endoscopy such as high-resolution endoscopy could lead to "stricter" indication criteria.

Advantage of endoscopic diagnosis

Some endoscopic findings have been reported to predict high-risk lesions for malignancy: (1) diameter > 20 mm; and (2) depressed macroscopic type[28]. Recently, Jung *et al*[29] reported that depressed type (OR, 4.1) and com-

Table 3 Histological follow-up studies of gastric intraepithelial neoplasia through mild to severe dysplasia

Reports (yr)	LGIN (including mild to moderate dysplasia)		HGIN (including severe dysplasia)	
	Detection of carcinoma *n* (%)	Interval (mean) *n* (%)	Detection of carcinoma	Interval (mean)
Saraga *et al*[49], 1987	1/64 (2)	4 yr	17/21 (81)	4 mo
Lansdown *et al*[46], 1990	0/7 (0)	-	11/13 (85)	5 mo
Rugge *et al*[50], 1991	12/69 (17)	1 yr	6/8 (75)	4 mo
Fertitta *et al*[51], 1993	7/30 (23)	10 mo	25/31 (81)	5 mo
Farinati *et al*[52], 1993	-	-	16/49 (33)[1]	-
Di Gregorio *et al*[53], 1993	6/89 (7)	2 yr	6/10 (60)	11 mo
Bearzi *et al*[54], 1994	8/81 (9.9)	-	27/44 (61)	-
Rugge *et al*[55], 1994	13/90 (14)	2 yr	14/18 (78)	9 mo
Kolodziejczyk *et al*[56], 1994	2/35[1] (5.7[2])	-	7/7 (100)	-
Kokkola *et al*[57], 1996	0/9 (0)	-	2/3 (67)	1.5 yr
Rugge *et al*[19], 2003	8/90 (8.9)	4 yr	11/16 (69)	34 mo
Yamada *et al*[58], 2004	0/38 (0)	-	1/10 (10)	54 mo
Park *et al*[59], 2008	3/26 (12)	58 mo[3]	1/1 (100)	58 mo[3]
Overall	60/628 (9.5)		145/231 (63)	

Proportion progressing to carcinoma and mean interval. [1]Moderate or severe; [2]Mild or moderate dysplasia; [3]Overall follow-up interval. LGIN: Low-grade intraepithelial neoplasia; IN: Intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia.

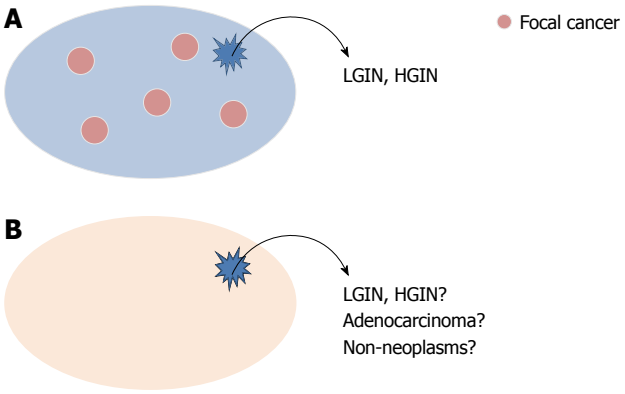

Figure 1 Reasons for the difficulty in making an accurate diagnosis based on a biopsy specimen. Cancer sometimes exists focally in the lesion and sampling error might occur (A); the structural atypia of both adenoma and well-differentiated adenocarcinoma is too subtle for small biopsy specimen. Moreover, regeneration of atypia induced by gastritis induces histological modification (B). LGIN: Low-grade intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia.

bined ulceration (OR, 5.6) were significant predictive factors correlated with cancer after endoscopic resection of gastric adenoma using multivariate analysis. For several decades, adenomatous polyps larger than 20 mm have been considered potentially malignant[30] and the rate of malignant transformations increased in accordance with an increase in the size. The depressed type is also reported to have more malignant potential. We, however, revealed that even patients with both smaller and elevated neoplasms had a greater than 40% under-diagnosis rate[27]. Similarly, cancer foci have been reported in hyperplastic polyps with a diameter of 5 mm[31]. Therefore, it is possible that a conventional endoscopic diagnosis based on size alone is not sufficient to make a precise preoperative diagnosis. Moreover, the surface appearance of an adenoma may also be an important factor for the diagnosis of malignancy[32].

Recent novel diagnostic modalities, including image-enhanced endoscopy, are useful for the differentiation of intraepithelial neoplasia[33]. Yao[33] reported that finding a white opaque substance on magnified endoscope with narrow band imaging differentiates intraepithelial neoplasia with a sensitivity of 94% and a specificity of 96%. Moreover, confocal laser endomicroscopy is reported to identify gastric superficial cancer/HGIN lesions with high validity and reliability compared to conventional white-light endoscopy and histological analysis for the final diagnosis[34]. Although image-enhanced endoscopy and confocal laser endomicroscopy are promising methods and modalities to improve the pre-therapeutic diagnostic accuracy of intraepithelial neoplasia, it is not yet clear whether they are clinically useful because of expert bias.

Treatment strategy for gastric intraepithelial neoplasia: follow-up or endoscopic resection?

There are two therapeutic principles for gastric intraepithelial neoplasias; one is to observe the intraepithelial neoplasia as a benign lesion unless biopsy specimens reveal an unequivocal malignant finding in consideration of the risk of treatment, and the other is to treat the intraepithelial neoplasia actively as a "diagnostic therapy". There are few guidelines to manage gastric intraepithelial neoplasia. Since 2000, the revised Vienna classification has helped to provide guidance for clinical management[17,21]. The category 4 lesions (high-grade dysplasia and intramucosal cancer) should be resected because they have a high potential for progression to adenocarcinoma[35]. On the other hand, there are no precise guidelines for the management of LGIN. Follow-up studies of HGIN reveal a striking high incidence, around 60%, of developing a carcinoma diagnosed within 1 year in the very short-term follow-up period (Table 3), supporting the validity of a treatment strategy for HGIN.

The natural course of gastric intraepithelial neoplasia

Table 4 Endoscopic submucosal dissection and endoscopic mucosal resection for early gastric cancer[60]

	EMR	ESD
Merits	Minimally invasive technique which is safe, convenient and efficacious	The advantage of achieving large en-bloc resections, not necessarily limited by lesion size
Demerits	Insufficient when treating larger lesions, especially larger that 15 mm	Requiring significant additional technical skills and a longer procedure time Prolonged learning curve
	High risks of local recurrence, especially when resections are not performed *en bloc* or when the resection margins are involved by tumor	A higher complication rate compared to standard EMR

ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection.

remains unclear. In particular, previous prospective long-term follow-up studies indicated that the gastric cancer incidence in LGIN ranges around 10% (Table 3). This low risk of malignant transformation compared to HGIN may be due to the slowly progressive natural course of LGIN and supports the follow-up strategy. Our current knowledge based on initial intervention, not follow-up, indicates that over 40% of LGIN is diagnosed as adenocarcinoma after resection[27]. This under-diagnosis rate may be higher than that in previous reports. In addition to optical advances in endoscopy, we speculated that the reason for our result was that we used endoscopic submucosal dissection (ESD), not endoscopic mucosal resection (EMR), because an ESD sample has an adequate tumor-free margin; in other words, it is easier to evaluate the atypical structures even if there is less nuclear atypia. Furthermore, we found two cases with submucosal cancers that required radical gastrectomy. These facts mean that even LGIN might not only be a premalignant lesion, but also a lesion that already contains cancer foci and a follow-up strategy might miss the chance for endoscopic therapy. Most LGIN progression to carcinoma, however, is generally low. Repeated endoscopic examination with biopsies burdens the patient with physiological, psychological and financial strains, although few reports discuss these points. Taken together, we consider that initial interventional strategy might be one option, even for LGIN if it is safer with an acceptable range and higher cost effectiveness.

EMR or ESD

Table 4 lists the merit and demerit of both EMR and ESD. EMR with a snare allows for a more accurate histopathological diagnosis than forceps biopsy because the lesion can be resected as a large piece. Previous prospective studies indicate that EMR provides higher diagnostic accuracy than forceps biopsy and the histopathology and complications are within expected norms; based on these studies, EMR is recommended by several reports for diagnosis[36-39]. EMR is limited, however, in that it sometimes results in a multiple piecemeal resection. Multiple piecemeal resection is associated with a specimen burning effect that interferes with an accurate pathological diagnosis. Additionally, a local recurrence may occur, with a reported incidence of approximately 10%[37]. The ESD-related complication rate is relatively low, based on a multicenter study of more than 1000 cases with gastric neoplasm[40].

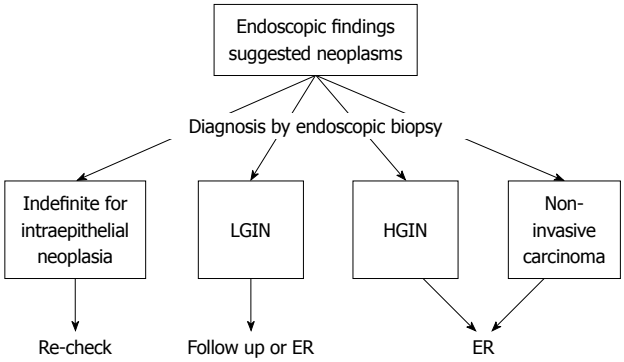

Figure 2 Treatment strategy for gastric non-invasive intraepithelial neoplasia diagnosed by endoscopic biopsy as a treatment flowchart (our opinion). LGIN: Low-grade intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia; ER: Endoscopic resection.

ESD allows for a more secure resection of larger lesions[41,42], resulting in a more accurate diagnosis because the margin of resected sample is larger than that for EMR. Although ESD requires greater skill, causes more complications, such as perforation, and has a longer procedure duration[41-44], in our recent study, the complication rate of ESD for gastric intraepithelial neoplasms was 5.4% for bleeding and 4.3% for perforation and the complete *en bloc* resection rate was 97%[27]. These rates are almost equivalent to those found in our multicenter study of more than 1000 cases with gastric neoplasm[40], although the perforation rate was only slightly more frequent than that reported for EMR. In detail, all patients with perforation were treated successfully with endoscopic clipping alone and the serious complication rate was only 0.45%. Therefore, the indication of ESD for LGIN, which is considered to be clinically less malignant, is controversial. Future use of ESD for LGIN requires further validation.

CONCLUSION

Endoscopic forceps biopsy is insufficient for a definitive diagnosis and therapeutic planning in patients with gastric intraepithelial neoplasia. Endoscopic resection should be considered as not only definitive treatment but also a procedure for a precise histological diagnosis for lesions initially assessed as gastric intraepithelial neoplasia by for-

ceps biopsy specimens. ESD may be a therapeutic option for gastric intraepithelial neoplasia for the purpose of total incisional biopsy. Finally, we have shown the treatment strategy for gastric non-invasive intraepithelial neoplasia diagnosed by endoscopic biopsy as a treatment flowchart (our opinion) (Figure 2). However, we still need to clarify the issue of evaluating the validity as to whether or not to follow-up or ER for LGIN diagnosed by endoscopic biopsy. A prospective study to clarify this is now planned.

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OBSERVATION

Oral refeeding in mild acute pancreatitis: An old challenge

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Abstract

Although the idea that pancreas rest has long been considered as a very relevant topic in acute pancreatitis (AP) therapy, the right time and type of diet to be offered to patients recovering from an acute attack are a great challenge to clinicians who treat this condition. Fortunately, the last decade was noted for several trials looking for the best answer to the question: "when and how to start oral refeeding in AP?" It is well known that 80% of patients present with mild disease characterized by usually uncomplicated clinical course are managed with pancreatic rest through nil per oral; while the use of specific nutritional intervention is an exception. Therefore, mild AP has been the most investigated form of AP and researchers have tried different kind of meals to offer calories and reduce costs by shortening hospitalization time. Usually in mild AP, the oral refeeding is introduced between the first 3 d and 7 d after hospitalization but, the type of diet and patients' tolerance have been scrutinized in detail with mixed results. Although 20% to 25% have pain recurrence requiring nutritional support and greater time of hospitalization, most patients seem to tolerate oral refeeding well. We propose analyzing the most recent investigations of this matter and their conclusions to develop a better understanding of the management of AP.

Key words: Acute pancreatitis; Oral refeeding; Nutrition; Treatment; Diet

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INTRODUCTION

Patients recovering from acute pancreatitis (AP) have been a great challenge for gastroenterologists with issues concerning the timing and type of diet to be offered without risk of increasing gland stimuli and worsening pancreas inflammation. The thought that as long as unfed they could be maintained through nil per oral was based on the idea that pancreas rest was considered an essential strategy in the treatment. This assumption has been taken up for years although there is, until now, little hard scientific basis to support it. It is conceivable that many patients with even more severe episodes of AP might eat quite early in the course of their disease, with no deleterious effects. In fact, nowadays there are no sufficient data to make clear this postulate. Moreover, this hypothesis was based only in physiological research. Until now no general consensus has been established about the type of diet and the appropriate time when it should be offered to patients convalescing from AP. An important aim in dealing with AP patients is to find a better way of managing oral food reintroduction. Since 80% of patients have mild AP, such patients have been more frequently investi-

gated. Pancreatic rest through nil per oral was always considered necessary in AP while the general rule suggested starting oral refeeding 3 d to 7 d after patients were hospitalized[1,2]. In general, oral intake of small amounts of calories have been usually considered the better strategy to start oral refeeding, once the clinical symptoms and signs of AP are absent, when the patients recover appetite and do not have nausea, vomiting or abdominal pain with normal bowel sounds[1,3]. Therefore, the traditional way to deal with this challenge during hospitalization was starting oral intake with clear liquids (CLD), since they do not exert relevant stimulatory effects on pancreatic exocrine secretion. A low-fat diet would be allowed to those who tolerate an initial trial[3]. Lipids can stimulate pancreatic secretion and clinicians have in mind that patients recovering from mild AP, eating a low-fat diet, have reduced exocrine pancreatic secretion as well as cholecystokinin (CCK) production. Therefore, potentially deleterious effects on the inflamed pancreas would occur, but there is little scientific evidence supporting this concept[4]. Although this approach is usually assumed, it is only based on clinical experience. New trials would be necessary to define amore appropriated diet to oral refeeding in patients recovering from AP.

REFEEDING IN AP

In 1997, Lévy *et al*[5], in a multivariate multicenter prospective study, tried to analyze the frequency and risk factors of recurrent pain during oral refeeding in 116 patients with mild AP. The conclusion was that pain relapse occurred in 20% of the patients and was more common in patients with necrotizing pancreatitis who had longer periods of pain. This survey seems to suggest that we would be able to predict high-risk patients who have pain recurrence during oral refeeding and could be considered a first step in pain relapse prevention. They also noticed that pain relapse frequency was not modified significantly in any of the therapeutic procedures adopted. However, in another evaluation, Chebli *et al*[6] studied 130 patients with mild AP and observed that during the oral refeeding period, 24.6% of patients had pain relapse, more frequently on days 1 (68.8%) and 2 (28%) and observed that it was related to higher serum levels of lipase on the day before refeeding, higher serum levels of C-reactive protein on the fourth day as well as peripancreatic fluid collections ($P < 0.01$). Pain relapse increased hospital stay and overall costs of disease treatment. Petrov *et al*[7] in a literature review, (cited in Cochrane Central Register of Controlled Trials, EMBASE, and MEDLINE) as well as the conclusions of abstracts of major gastroenterological meetings, taking into account that outcome measures studied were the incidence of pain relapse and length of hospitalization (LOH), pointed that only three studies met the inclusion criteria. They concluded that sixty (22%) of 274 patients had pain relapse during the course of AP and in 78.3% it occurred within 48 h after starting oral refeeding. All three studies found a difference in scores of severity in patients who had or had not pain relapse and found a significant increase in the LOH in those whose pain reappeared after oral refeeding. Therefore it becomes clear that, in these patients, there is an increased cost of treatment. Jacobson *et al*[8] developed a prospective randomized trial comparing CLD *vs* low-fat solid diet (LFSD) as the initial meal in mild AP. They randomized two similar groups with mild AP and hypothesized that initiating oral nutrition with LFSD after mild AP would be well-tolerated and would result in a shorter LOH. Their final conclusion was that starting oral nutrition after mild AP with a LFSD appeared to be safe and provided more calories than a CLD, but did not result in a shorter LOH. On the other hand, Reber[4] considered that earlier discharge was not an advantage and suggested a flaw in trial design, because he found it intuitively obvious that at least 1 d of hospitalization would have been saved in those who had begun with solid food. Moreover, Reber[4] posed two interesting questions: whether offering solid food to patients with AP can be done safely, does the nutrient composition (in particular, the fat content) of the diet affect the clinical response; and is it more relevant to feed such patients with a low rather than a higher fat content that might be more palatable? Meanwhile, Sathiaraj *et al*[9] compared two groups of mild AP patients who, as their initial meal had a soft diet or a clear liquid diet. They observed a statistically significant decrease in the LOH (total and post-refeeding) of a median 2 d in patients receiving a soft diet ($P < 0.001$) but no significant difference for refeeding interruption due to pain, was observed between the two groups, and patients who started on a soft diet consumed much more calories and fats on study day 1 ($P < 0.001$). The researchers conclusion was that oral refeeding with a soft diet in patients with mild AP can be considered safe and can result in shorter LOH. Studies of the best feeding option has come from comparisons between CLD and LFSD. Different kinds of diets have been tried but no one study was ambitious enough to investigate the tolerance of a full solid diet as the initial meal in AP. In 2010, Moraes *et al*[10] conducted an investigation to try to demonstrate whether or not a heavier diet could be dangerous given the evidence of previous studies. They observed through a prospective, randomized, controlled double blind clinical trial that oral refeeding, with a full solid diet in mild AP, was well tolerated by most patients and resulted in a shorter LOH among patients without abdominal pain relapse. Therefore, employing this strategy would save health care resources. It was observed that a full solid diet may be more palatable and a cost-saving alternative for the dietary management of patients recovering from mild AP. The authors also called attention to the fact that their findings may not be applied to patients with severe AP, since only those with mild AP were evaluated. As far as we are concerned, this is a highly relevant contribution to the optimal dietary approach to oral refeeding in mild AP but further clinical trials are necessary to investigate other strategies to prevent pain relapse during oral refeeding in patients with AP. It is well known that a 20% intolerance can be seen in AP, regardless of the type of

initial oral refeeding diet.

After analyzing the data in recent studies reviewed above, we can state that in patients with mild AP, the nutrient composition and the physical features of meals do not appear to change the clinical course of disease. Hence, a very interesting question arose: would we be able to match the interesting clinical data to those found in physiological studies[11,12] that show slower pancreatic response and higher pancreatic enzyme output in response to a high-fat solid diet with greater caloric loads? It is possible that the injured pancreas can have an attenuated response to feeding stimuli. So, we can speculate that the basal and stimulated pancreatic enzyme secretion, in particular pancreatic secretory response to CCK, may be woken markedly early, after the onset of AP, as have been shown in experimental studies[13,14]. Evidence allows us to conclude that in human pancreatitis the injured pancreas may be less responsive to stimulation by food than previously considered[15,16].

In the last two decades we noticed that several investigations tried to test the tolerance to food stimulation of the inflamed gland after an episode of mild AP. Initially on a longer fasting period followed by an early oral refeeding with clear liquid and a low fat diet. Moreover, the evidence that a full solid meal can be used in such circumstances with better tolerance, demonstrated that increased nutritious calories and a reduction in therapy costs can occur. However, the findings of Moraes *et al*[10] lead us to suppose that we have now found the answer to an old question. A full solid diet is well tolerated by the majority of patients and results in a shorter length of hospital stay without abdominal pain relapse, thereby saving health care resources. Petrov[17] pointed out in Moraes study that the rate of feeding intolerance was around 20%, regardless of the type of initial diet used for oral refeeding and this was unacceptable both from the perspective of patient's quality of life and the cost of treatment. However, this rate of intolerance was observed also in other trials[5,6] and it seems a rule in oral refeeding of patients with mild AP.

CONCLUSION

In summary, identifying patients who are at high risk of developing pain recurrence during oral refeeding due to more intense or persistent pancreatic inflammation on the day before refeeding (for example, those patients presenting with a substantially increased serum lipase concentration and high level of C-reactive protein)[6], might allow a timely implementation of more specific therapeutic measures for this subgroup of patients such as nasojejunal tube feeding. Thus further clinical investigations are necessary to improve the identification of these subgroup patients and to establish an adequate strategy to prevent their pain relapse. To the best of our knowledge it seems that when considering full solid diet refeeding, we do not need to be afraid of "wakening the sleeping tiger. " The in-hospital time can be, saving costs.

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Role of Sonic Hedgehog signaling during progression from inflammation to cancer in the stomach

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Abstract

Despite advances in treatment and the declining incidence, gastric cancer remains the second leading cause of cancer-related deaths in the world. Understanding the progression from inflammation to cancer in the stomach is crucial in the development of novel therapies and strategies for treating this disease. Chronic inflammation of the stomach is typically caused by *Helicobacter pylori* (*H. pylori*) and resulting lesions may lead to gastric cancer. During the progression from inflammation to cancer, the stomach epithelium changes with evidence of the disruption of normal epithelial cell differentiation and infiltrating inflammatory cells. Coincident with the development of atrophic gastritis and metaplasia, is the loss of the gastric morphogen Sonic Hedgehog (Shh). Given its critical role as a regulator of gastric tissue homeostasis, the disruption of Shh expression during inflammation correlates with the loss of normal epithelial cell differentiation, but this has only recently been rigorously tested *in vivo* using a unique mouse model of targeted gastric Shh deletion. While pre-neoplastic lesions such as atrophic gastritis and intestinal metaplasia are associated with the loss of Shh within the acid-secreting glands of the stomach, there is a clear link between elevated Shh and signaling to gastric cancers. The current review focuses on the effects of aberrant Shh expression and its role in the development of gastric cancer, specifically in response to *H. pylori* infection.

Key words: *Helicobacter pylori*; Interleukin-1β; Acid secretion; Gastric tissue homeostasis

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INTRODUCTION

Despite advances in treatment and declining incidence, stomach cancer remains the second leading cause of cancer-related deaths in the world[1]. Understanding the progression from inflammation to cancer in the stomach is crucial in the development of novel therapies and strategies for treating this disease. The Hedgehog family of proteins, comprised of Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), has been implicated in a variety of solid tumors including stomach cancer[2-4]. This review will focus on the role of Shh in the progression from inflammation to tumorigenesis of gastric carcinomas and its role in stomach cancer.

Hedgehog was given its name from the "spiny" phe-

notype found in *Drosophila* embryos with a mutation in the gene[5]. Three mammalian homologs have since been discovered: Shh, Ihh and Dhh. Ihh and Dhh have mostly tissue-specific expression while Shh has widespread expression of which deficiency leads to neural, limb growth and foregut defects[6]. Hedgehog is generated as a preprotein before its signal sequence is cleaved, yielding an approximately 45 kDa precursor. The precursor is internally cleaved and a cholesterol moiety is attached to a glycine residue on the nascent carboxy-terminus[7]. This covalent attachment of cholesterol is a unique modification among secreted ligands and is a requirement for tissue specificity and localization of the molecule. In vertebrates, Shh binds and inhibits Patched-1 (Ptc), consequently activating Smoothened (Smo), which transduces the Hedgehog signal into the cytoplasm, activating the Gli family of transcription factors[8,9]. Despite this information, the events leading to Gli activation, especially in the mammalian stomach, are poorly understood.

Shh is a peptide morphogen produced in gastric epithelium; however, expression in specific cell types has been controversial. Recently, a study utilizing a Shh-LacZ reporter mouse line identified all major cell lineages of the corpus expressing Shh; these included surface pit, mucous neck, zymogenic and parietal cells[10]. Some stromal cells express signal transduction components in addition to Shh; these include the 12-transmembrane receptor, Ptc, Smo, 7-transmembrane receptor and Gli transcription factor family. The Hedgehog ligand activates Smo which leads to transcriptional activation of Gli1, Gli2 becoming an activator and Gli3 no longer acting as a repressor[11]. Therefore, Hedgehog signaling results in an increase of activator Gli and a decrease in repressor Gli. These events are crucial in the development and maintenance of multiple organs and the role of Shh as a key morphogen has been well documented[5,12].

Shh has important roles in the development of multiple organ systems including neuronal and gastrointestinal systems[13,14]. Shh in the mammalian stomach has been found to be hormonally regulated by gastrin and requires an acidic environment for proper processing[15]. The acid-activated pepsin A protease was found to mediate the processing of the 45kDa precursor molecule to the active Shh molecule. These findings indicate the importance of the acidic gastric environment for maintenance of Shh in the stomach.

Much of what we know about Shh's role in mammals has come from studies in animal models. Shh-knockout mice display key features that support the importance of proper Shh homeostasis for normal development and physiological function.

Shh -/- mouse embryos displayed multiple gut abnormalities including overt gut malrotation and intestinal transformation of the stomach[16]. Additionally, neurons of the enteric nervous system underwent abnormal differentiation compared to wildtype. While this system provided evidence for the role of Hedgehog in the coordination of developmental genes, these mice die at or shortly after birth.

In order to study the effects of Shh signaling in the stomach, Xiao *et al*[17] developed a mouse model expressing a parietal cell-specific deletion of Shh (HKCre/Shh^{KO}). This study found evidence of hypochlorhydria, hypergastrinemia and foveolar hyperplasia by 8 mo of age. Additionally, a delay in the differentiation of zymogen cells, demonstrated by an increase in immature cells expressing markers for both mucous neck and zymogen cells, was documented in the stomach of HKCre/Shh^{KO} mice[17]. The deletion of Shh from parietal cells led to alterations in stomach morphology and gastric cell differentiation; however, there was no evidence of parietal cell atrophy, a key step in the development of stomach cancer in *Helicobacter pylori* (*H. pylori*) infection[18,19]. The absence of parietal cell atrophy suggests that the loss of Shh expression alone is not sufficient to trigger the development of stomach cancer.

H. PYLORI INFECTION AND SONIC HEDGEHOG

H. pylori infection is globally widespread and a major cause of chronic atrophic gastritis with persistent infection in 50% of the global population[20,21]. While infection with *H. pylori* has been directly linked to the development of gastric cancer, the mechanism of tumorigenesis is still unclear. There are many factors that have been considered in *Helicobacter*-mediated carcinogenesis; however, the focus of this review will be to discuss its contribution by modulating Shh expression. *H. pylori* infection primarily contributes to gastric cancer development through two mechanisms: loss of Shh by destruction of parietal cells and induction of a chronic inflammatory gastric environment.

H. pylori and the inflammatory response

The ability of *H. pylori* to evade the primarily Th1-mediated host immune response contributes to the development of chronically elevated interleukin-1β (IL-1β) and other inflammatory cytokines in the stomach[10,22]. A study of *H. pylori* associated with chronic atrophic gastritis found upregulation of a gene encoding cysteine-rich protein A, which induces Th1 cytokines interferon (IFN)-γ and IL-12[23]. Additionally, *H. pylori*-associated atrophic gastritis has been found to be more frequent in patients with pro-inflammatory polymorphisms of genes for IL-1β and tumor necrosis factor (TNF)-α[24,25]. Specifically, it has been shown that elevated IL-1β leads to the suppression of Shh, which is necessary for the growth and differentiation of the gastric mucosa during cell restitution during *H. pylori* infection[10,26].

Loss of Shh during chronic inflammation

The loss of parietal cells through *Helicobacter* infection and subsequent autoimmunity has been well documented[22,27]. Molecular mimicry between the H^+,K^+-ATPase local-

ized to parietal cells canaliculi and *H. pylori* leads to the selective destruction of parietal cells during the immune response to infection. There is evidence that Shh is expressed and secreted from parietal cells and that the loss of parietal cells results in lower expression of Shh[15,28,29]. Additionally, the higher luminal pH in the stomach during *Helicobacter* infection may interfere with Shh signaling as it has been shown that gastric Shh processing is pH-dependent[15]. The loss of Shh, coupled with an increase in the proliferation and differentiation of gastric stem cells, contributes to improper regeneration of gastric epithelium. Shh acts *via* an autocrine loop in the stomach and is implicated in stem cell restitution of damaged gastric mucosa during *H. pylori* chronic infection[30]. Despite its crucial role in gastric homeostasis, the loss of Shh alone is not sufficient for neoplastic transformation[17]. An additional study examining the role of Shh in the presence of *Helicobacter* demonstrated the importance of the chronic inflammatory environment induced by infection for the induction of gastric atrophy associated with Shh suppression[10].

H. pylori infection in Mongolian gerbils has been found to induce inflammation and a loss of Shh expression[31-33]. Infection resulted in a smaller region of Shh-expressing cells in the stomach and hyperproliferation of Shh-negative cells after 51 wk. In humans, the loss of Shh expression coincided with the induction of caudal type homeobox 2 (Cdx2), intestine-specific transcription factors in the stomach[34]. In fact, Cdx2-transgenic mice developed gastric polyps with invasive gastric adenocarcinoma, implicating intestinal metaplasia in gastric carcinogenesis[35]. Further studies of this mouse model demonstrated Cdx2 transcriptionally down-regulating Shh through its promoter, inducing metaplasia through the expression of intestinal metaplastic mucosa and loss of gastric phenotype[35]. Cyclopamine, an inhibitor of Shh signaling, has been shown *in vivo* to reduce the expression of parietal cell-specific H^+,K^+-ATPase[36]. The lack of an appropriate level of Shh signaling leads to improper gastric morphogenesis during healing and ultimately gastric mucosal atrophy, predisposing the individual to gastric cancer[30].

ROLE OF SONIC HEDGEHOG IN THE DEVELOPMENT OF GASTRIC CANCER

With an estimated annual 989 600 new cases and 738 000 deaths, stomach cancer is the second leading cause of cancer-related deaths worldwide[1]. The importance of Shh in the development and maintenance of proper gastric function is established; however, there are alternative explanations for its role in carcinogenesis.

Increased Shh from stomach cell selection

The aforementioned studies hypothesize that lower Shh expression in *H. pylori* infection leads to impaired differentiation and morphogenesis during healing secondary to inflammatory signals. Other studies suggest *H. pylori* infection selects for cells that are resistant to cell death and that these cells produce higher levels of Shh[37,38]. The anti-apoptotic properties of these cells may contribute to the tumorigenesis in these human gastric carcinoma cells[38]. It is important to consider that an *in vitro* study may not be the most appropriate representation of the development of human disease due to the lack of environmental factors, including inflammatory cytokines, signaling and morphogenic factors from parietal cells, and the contribution of progenitor cells.

Contribution of CD44+ cells as cancer stem-like cells

Previous data suggests the involvement of CD44+ cells in the repair of gastric mucosa in *H. pylori* infection[39]. In fact, this study found that both IFN-γ and TNF-α increased CD44 expression; however, elevated levels of IL-1β and IFN-γ may cause transformation of some of these recruited stem cells into cancer stem cells (CSCs). CD44 has been identified as a marker of CSCs, thus supporting the idea that gastric CSCs exist and possess tumorigenic properties both *in vitro* and *in vivo*. In fact, Takaishi *et al*[40,41] found that CD44 expression correlated with dysplastic gastric glands and invasive gastric lesions in *Helicobacter*-infected mice. In a mouse model of activated gastric Wnt and prostaglandin E2 signaling, CD44 was upregulated in gastric tumors[42]. Wnt signaling is a downstream effector of Hedgehog signaling; therefore, this finding provides additional evidence that CD44 may play a role in gastric tumorigenesis, possibly through Shh signaling[43,44]. In another study, CD44+ cells isolated from these tumors had high expression of CD133, another marker used in identifying CSCs, and low expression of MUCA5C, a marker of gastric epithelial differentiation[42,45]. Furthermore, Shh has been found to be upregulated in CD44+ cells and inhibition of Shh signaling by cyclopamine blocks the chemoresistant and self-renewing properties of these proposed CSCs[46]. In humans, CD44+ tumors are also associated poorer survival in patients with intestinal-type gastric adenocarcinoma[47].

Reappearance of Shh during cancer development - role of bone marrow-derived mesenchymal stem cells

The seemingly paradoxical disappearance and reappearance of Shh expression during gastric carcinogenesis requires further elucidation. The loss of parietal cells during *H. pylori* infection may explain the initial deficit of Shh expression, while the repopulation of the gastric mucosa by stem cells expressing Shh may explain the high levels of expression found in gastric adenocarcinomas. Of particular interest are bone-marrow-derived mesenchymal stem cells (MSCs) and their potential role in tumorigenesis. These cells are recruited to sites of tissue injury and have been hypothesized to be the link between chronic inflammation and stomach cancer[48,49]. Houghton *et al*[48,49] transplanted marrow-derived cells tracked by X-galactosidase in a lethally irradiated mouse model of chronic *Helicobacter* infection. They found that the mice developed metaplasia and dysplasia, and that the marrow-derived cells were pro-

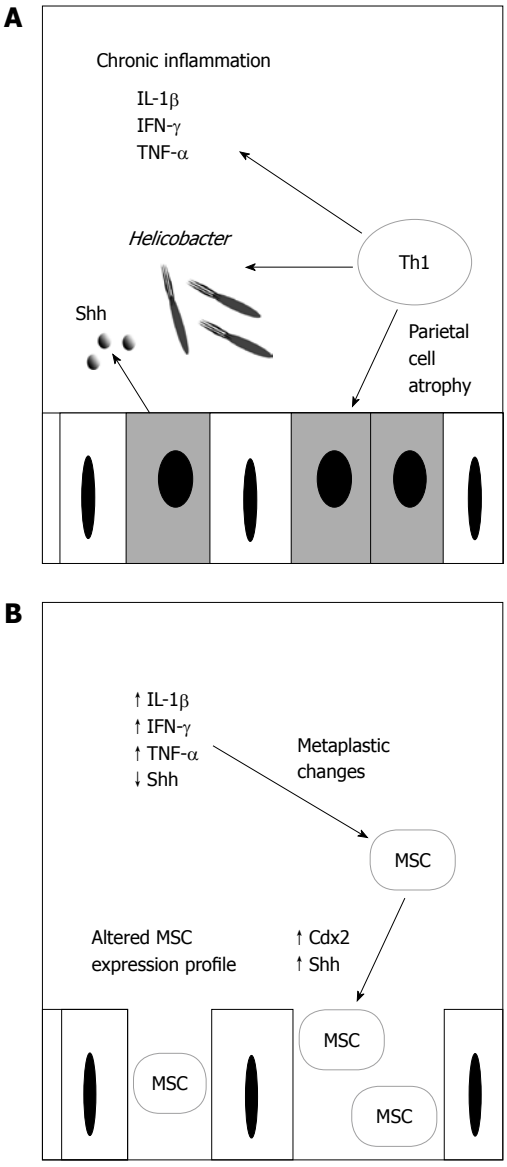

Figure 1 Neoplastic transformation of the stomach triggered by chronic inflammation. A: *Helicobacter* infection induces chronic inflammation mediated by Th1 cells leading to loss of Shh-secreting parietal cells; B: Inflammatory cytokines present in stomach influence expression of recruited MSCs, altering their expression profile leading to metaplastic changes, the preliminary step in gastric carcinogenesis. IL-1β: Interleukin-1beta; IFN: Interferon; TNF: Tumor necrosis factor; Shh: Sonic hedgehog; Cdx2: Caudal type homeobox 2; MSCs: Mesenchymal stem cells.

liferating in the dysplastic glands of the stomach. Furthermore, this MSC engraftment could only be demonstrated with concurrent *Helicobacter* infection, supporting the idea that chronic inflammation is necessary for the development of parietal cell atrophy and subsequent neoplastic transformation. Furthermore, it has been shown that inhibition of Hedgehog signaling by cyclopamine decreases MSC cell proliferation and clonogenecity, suggesting a role of Hedgehog in the maintenance of this cell population in the periphery[50]. Emerging data provides further evidence for the role of MSCs in gastric carcinogenesis. MSCs have been isolated and characterized from patients undergoing radical gastrectomy for stomach cancer[51,52]. These cells expressed CD44, had a larger population in S phase compared to control, but lacked tumorigenic properties when transplanted into BALB/c nude mice. Further studies are warranted on the impact of aberrant Shh expression on the differentiation of recruited MSCs and their role in gastric tumorigenesis.

Persistent *H. pylori* infection remains one of the key events leading to gastric adenocarcinoma. The loss of parietal cells and secretion of inflammatory cytokines, including IL-1β and IFN-γ secreted by Th1 cells induced by persistent *Helicobacter* infection, causes suppression of Shh signaling (Figure 1A). When recruited MSCs repopulate the gastric epithelium, the presence of inflammatory cytokines in combination with the absence of adequate Shh expression allows for metaplastic changes, including increased expression of Cdx2, ultimately leading to dysplasia and subsequent cancer development (Figure 1B). The malignant transformation of MSCs into cancer-promoting cells may be induced by inflammatory cytokines secreted during chronic gastritis. Malignantly transformed MSCs may be the site of Shh secretion associated with gastric carcinomas. However, the *in vivo* malignant transformation of MSCs in response to chronic inflammation is not yet known.

CONCLUSION

Chronic inflammation is typically caused by *H. pylori* and is the most consistent lesion leading to gastric cancer. During the progression from inflammation to cancer, the stomach epithelium changes with evidence of the disruption of normal epithelial cell differentiation, infiltrating inflammatory cells and the recruitment of bone marrow derived MSCs. Coincident with changes in cell differentiation associated with the development of atrophic gastritis and metaplasia, is the loss of Shh. Given its predicted critical role as a regulator of gastric tissue homeostasis, the disruption of Shh expression during inflammation would be expected to result in loss of normal epithelial cell differentiation, but this has only recently been rigorously tested *in vivo* using the HKCre/Shh^{KO} mouse model. Studies using HKCre/Shh^{KO} mice reveal for the first time a direct role of Shh as a regulator of epithelial cell function and differentiation in the normal adult stomach. The dysregulation may be seen as a global increase or decrease in expression, or in altered location of expression. For example, atrophic gastritis and intestinal metaplasia are associated with the loss of Shh within the fundic mucosa[18,19], while elevated Shh and signaling is associated with gastric cancers[37,46,53]. Although the association between Shh and gastric cancer is clear, the mechanism that regulates the production of Shh protein within the tumor microenvironment and the precise role of Shh in tumor progression are still largely unknown. Understanding the role of Shh in the neoplastic transformations associated with chronic gastric inflammation would allow for the development of targeted therapeutics and preventative strategies.

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Gastric mammalian target of rapamycin signaling, hormone production and energy metabolism

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Abstract

The obesity epidemic imposes a significant health burden on human beings. Current understanding of the mechanisms underlying the development of obesity is incomplete and contemporary treatment is often ineffective. Gastrointestinal hormones are important regulators of food intake and energy metabolism. Previous studies indicate that the mammalian target of rapamycin signaling pathway in the gastric mucosa is crucially involved in fuel sensing in the gastrointestinal tract and plays a critical role in the coordination of nutrient availability and ingestive behavior *via* the production of gastric hormones. As an important component of the brain-gut axis regulating food intake and energy homeostasis, energy sensing in the gastrointestinal tract may provide a novel insight into our understanding of the precise coordination between the organism and cellular energy state.

Key words: Gastric mammalian target of rapamycin; Hormones; Energy metabolism

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INTRODUCTION

Food intake and energy metabolism are regulated by the reciprocal actions of a group of anorexigenic peptides, which include leptin, insulin, cholecystokinin, peptide YY and glucagon-like peptide, and by the actions of a group of orexigenic peptides, including ghrelin. The majority of these hormones are secreted by endocrine cells scattered throughout the gastrointestinal tract[1]. All these hormones are proposed to modulate the activity of the energy metabolism center within the hypothalamus, ultimately leading to a change in feeding behavior and the control of metabolic homeostasis[2]. While many studies reveal that nutrient sensing molecules within the hypothalamic neurons are critical in the control of energy homeostasis[3] and defects in fuel sensing at the hypothalamic cellular level may lead to energy imbalance at the organism level and to the development of obesity[4], a recent study suggests that there also exists a fuel sensing mechanism in the gastric mucosa[5]. This finding suggests that the

interaction between peripheral and central fuel sensing mechanisms is a crucial feature of feeding behavior and energy homeostasis[5]. The peripheral fuel sensing mechanism in the gastric mucosa may function to regulate the production of gastric hormones and therefore contribute to the modulation of energy metabolism.

FUEL SENSING MECHANISM

Obesity is defined as the condition in which energy intake consistently outpaces energy expenditure leading to the accumulation of excess fat to an extent that health is negatively affected. The development of obesity is linked to small but cumulative discrepancies between caloric intake and energy expenditure[6]. Under normal conditions, balance in energy metabolism is maintained by a precise regulation of cellular activity in multiple organs that matches nutrient supply at the organism level[7]. The link between the energy status of individual cells and the overall energy balance of the entire organism is complex and remains largely unknown. Many studies have identified the hypothalamus as a critical organ for integrating intracellular metabolic processes with energy homeostasis at the organism level[7], adjusting food intake to match the level of overall cellular activity. Recent investigations have identified 5' AMP-activated protein kinase (AMPK)[8] and mammalian target of rapamycin (mTOR)[9] as key fuel sensors in hypothalamic neurons. AMPK is a serine-threonine protein kinase which serves as a cellular fuel sensor to protect cell viability in response to ATP depletion[10]. AMPK is tightly regulated, monitoring changes in the cellular ratio of adenosine monophosphate (AMP) and adenosine triphosphate (ATP). Recent studies have suggested that AMPK in the hypothalamus regulates energy metabolism by integrating inputs from multiple peptide hormones, neurotransmitters and nutrients. Alteration of hypothalamic AMPK activity leads to change in food intake and body weight[11-13]. mTOR, a highly conserved serine-threonine kinase, has been reported to serve as an intracellular ATP sensor. *In vitro* studies have demonstrated that cellular levels of ATP regulate mTOR signaling[14]. Aberrant mTOR activity is linked to the development of cancer, diabetes and obesity[15]. Significant elevation of mTOR signaling has been observed in liver and skeletal muscle of insulin-resistant obese rats maintained on a high fat diet[16]. In contrast, absence of the mTOR downstream target, S6 kinase 1, protects against diet-induced obesity and improves insulin sensitivity in mice[17]. mTOR signaling in hypothalamic neurons is involved in neuronal sensing of nutrient availability and regulates food intake and energy balance[9]. These observations suggest that mTOR plays an important role in central neuronal control of nutrient intake and energy balance. Further studies indicate that mTOR signaling is a potential downstream pathway for food intake regulation in response to hypothalamic AMPK[18], likely through the mediation of tuberous sclerosis complex 2, a known inhibitor of mTOR signaling[19]. Thus, food intake and nutrient metabolism may be coordinately regulated by linking AMPK and mTOR signaling pathways in the hypothalamus. These observations have motivated extensive studies of hypothalamic fuel sensing mechanisms and hypothalamic regulation of energy metabolism[7]. In contrast, virtually no attention has been focused on fuel sensing by the gastrointestinal tract, despite its critical role in the regulation of food intake.

GASTRIC mTOR IS A FUEL SENSOR INTEGRATING FUEL SUPPLY WITH HORMONE PRODUCTION

A series of studies have identified mTOR as a potential candidate of fuel sensor in the gastric mucosa because of its expression in a distinct group of the gastric endocrine cells, its reciprocal relationship with energy status and its role in the regulation of gastric hormone production[1,5,20].

Co-localization of mTOR signaling molecules in gastric neuroendocrine cells

Chromogranin A is a widely recognized marker of neuroendocrine cells, including those of the stomach, large and small intestine, adrenal medulla and pancreatic islets[21]. It is also an excellent marker for neuroendocrine tumors[22]. In the gastric fundus, the active forms of mTOR signaling molecules express in cells located in the basal one third of the gastric mucosa. One third of chromogranin A-immunoreactive cells express phospho-S6K1, the downstream target of mTOR. The majority of the mTOR positive endocrine cells are ghrelin positive with a small fraction of cells stained positive for gastrin immunoreactivity. No mTOR signaling molecule is located within somatostatin immunoreactive cells[20]. These studies suggest that mTOR signaling may selectively influence the function of a subpopulation of gastric endocrine cells.

A reciprocal relationship between gastric mTOR signaling and energy status at the organism level

Gastric mTOR signaling also senses the body energy status. Gastric mTOR activity decreases in 48 h fasted mice relative to fed animals. In contrast, there is a significant increase in gastric phospho-mTOR (Ser2448) and phospho-S6 (Ser235/236) expression in obese mice relative to lean animals[5]. Gastric mTOR signaling is therefore reciprocally related with the short- and long-term changes in nutritional status at the organism level.

Gastric mTOR and hormone production

Numerous peptides are synthesized and released from distinct populations of secretory neuroendocrine cells throughout the gastrointestinal tract[23]. Their roles in the regulation of gastrointestinal function have been well characterized for many years and it is now becoming evident that they also modulate feeding behavior and energy metabolism *via* distinct mechanisms. Major neuroendocrine products have been identified as gastrin in G

cells, histamine and uroguanylin in enterochromaffin-like (ECL) cells, somatostatin in D cells, serotonin in EC cells and ghrelin in X/A-like cells[23,24]. Hormones secreted from gastric endocrine cells bind to receptors located in the hypothalamus to regulate food intake and energy metabolism[1]. The fuel sensing mechanism is critical for the regulation of gastrointestinal hormone synthesis and secretion and therefore provides a fine tuning for the peripheral and central control of feeding behavior and energy homeostasis.

Ghrelin: In 1999, ghrelin was isolated from the human and rat stomach as the endogenous ligand for the growth hormone secretagogue-receptor (GHS-R)[25]; it is synthesized mainly by X/A-like cells in the gastric mucosa and secreted into the circulation[26]. Several molecular forms of ghrelin are found in the stomach and circulation: the 28 amino acid ghrelin with n-octanoylated serine in position 3; des-acyl ghrelin, an identical peptide in which the third amino acid serine is not acylated; and the 27 amino acid des-glutamine 14 ghrelin produced by alternative splicing of the ghrelin gene[24]. Another putative proghrelin peptide, termed "obestatin", has been proposed[27] but biochemical and functional evidence supporting its existence has not been forthcoming. Octanoylation is necessary for ghrelin to bind with its receptor, GHS-R. Ghrelin-O-acyltransferase, the enzyme responsible for ghrelin acylation, has been recently characterized as a member of the Membrane Bound O-Acyltransferases family[28,29]. Ghrelin has been reported to exercise a broad array of functions including control of food intake[30] and glucose metabolism[31]. Exogenous ghrelin induces adiposity in rodents by stimulating an acute increase in food intake, as well as a reduction in fat utilization[32]. Blocking the action of ghrelin by either its receptor antagonism[33] or interfering with its availability for its receptor by neutralizing antibodies[34] or Spiegelmer RNA[35] have been reported to show some effects on reduction of food intake and body weight, although the immunization against ghrelin fails to cause long-term body weight reduction. Ghrelin exerts its orexigenic effect *via* a mechanism involving the central nervous system; at least part of the orexigenic effect of ghrelin is mediated by up-regulating the genes encoding orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP)[36] in the hypothalamus. During fasting, ghrelin secretion increases[37]. Conversely, plasma ghrelin concentration decreases in most obese subjects[38] except in Prader-Willi syndrome[39]. Ghrelin and its receptor are expressed in human and rat pancreatic islets[40]. Ghrelin inhibits glucose stimulated insulin secretion in a dose-dependent manner *in vitro*[41]. Intravenous ghrelin injection decreases plasma insulin and increases plasma glucose levels, likely by inhibition of insulin secretion[41]. Absence of ghrelin in ob/ob mice lowers blood glucose substantially even though it does not decrease food intake or body weight[42].

The secretion of ghrelin is tightly coupled to the fasting or fed state[43]. While it is presumed that precise control in the production and secretion of ghrelin is critical for the maintenance of energy balance, the molecular mechanisms by which ghrelin producing cells modulate transcription and translation of ghrelin to match overall energy status remain largely unknown. A recent study has demonstrated that gastric mTOR is a critical molecule coordinating the ghrelin production with energy supply levels. In gastric mucosa, mTOR signaling molecules are located mainly in the ghrelin-positive cells. More than 90% of ghrelin-positive cells stain positively for mTOR signaling molecules. There exists a reciprocal relationship between gastric mTOR signaling and the expression and secretion of ghrelin during changes in energy status. Inhibition of gastric mTOR signaling increases expression of gastric ghrelin and circulating ghrelin. Conversely, activation of gastric mTOR signaling attenuates the expression and secretion of ghrelin. All these data support the concept that gastric mTOR activity is reciprocally linked to the production of ghrelin[5].

Gastrin: Gastrin is an acid secretagogue peptide discovered by Edkins[44] in 1906. Gastrin stimulation of ECL cells results in the increased synthesis and release of histamine, which then induces acid secretion by binding to the H receptors located on parietal cells[45]. Other major physiological functions of gastrin on the gastrointestinal tract includes functioning as a growth/differentiation factor[46]. Gastrin release is stimulated by vagal impulses during the cephalic phase and by intramural neural reflexes as well as by the presence of food constituent in the gastric lumen during the gastric phase of acid secretion[47]. Increased production of hydrochloric acid lowers intragastric pH and inhibits further secretion of gastrin[48]. Gastric mTOR signaling may be involved in the regulation of gastrin synthesis and secretion in a proportion of gastric G cells. Only 1/3 of gastrin cells contain mTOR signaling molecules, suggesting that regulation of gastrin synthesis and secretion may involve multiple mechanisms[20].

Somatostatin: Somatostatin was originally isolated as a hypothalamic somatotropin-release inhibiting factor[49] and was soon found to potently inhibit the secretion of multiple hormones, including gastrin[50]. However, production of somatostatin appears not to be affected by gastric mTOR. No mTOR activity is detected in somatostatin positive cells. Furthermore, inhibition of gastric mTOR signaling by rapamycin demonstrates no effect on the synthesis and secretion of somatostatin[20].

All of this evidence supports that mTOR signaling selectively modulates the production of gastric hormones. The differential regulation of gastric hormones by mTOR signaling may provide an alternative strategy for the development of novel therapeutics for obesity and other disorders of energy metabolism.

GASTRIC FUEL SENSING AND ENERGY METABOLISM

In the central nervous system, fuel substrates such as glu-

cose, fatty acids and amino acids, or hormones including leptin and insulin, act on the hypothalamic neurons to inform the energy metabolism regulating center of the energy status[3]. Specific populations of "glucosensing" neurons have been identified[51]. In the hypothalamic arcuate nucleus, pro-opiomelanocortin neuron is the glucose-excited neuron, while NPY neuron is inhibited by glucose. These neurons form the neuronal circuits to monitor and integrate the quantitative and temporal changes in glucose concentration[51]. By regulating their activity and neurotransmitter release, these neurons coordinate the central glucose level with the peripheral glucose production and utilization to maintain the glucose homeostasis[3,51].

How hypothalamic neurons sense the energy supply is being actively explored. Studies by Cota *et al*[9] strongly support the notion that mTOR is a critical intracellular molecule within hypothalamic neurons to coordinate the energy supply with food intake and energy metabolism. Although mTOR and S6K1 are widely expressed in a variety of tissues within the CNS, the phosphorylated form of these two kinases is abundantly localized in the hypothalamus, particularly in the NPY/AgRP neurons. Activity of the mTOR pathway in the hypothalamus is tightly linked with energy supply. mTOR activity decreases during fasting and its activity conversely increases during re-feeding. Central administration of leucine, a branch chained amino acid, decreases food intake and body weight by activation of the hypothalamic mTOR signaling. Leptin stimulates hypothalamic mTOR activity and inhibition of mTOR signaling blunts the anorectic effect of leptin[9]. Hypothalamus specific expression of dominant negative S6K results in an increase in food intake, whereas expression of constitutively active S6K decreases food intake[52]. These observations suggest that mTOR is a critical fuel sensor in the hypothalamus.

Inhibition of mTOR signaling by rapamycin has been demonstrated to increase food intake. Such an orexigenic effect of rapamycin may be mediated by ghrelin. Intraperitoneal injection of rapamycin stimulates ghrelin secretion and expression. Ghrelin receptor antagonist D-Lys-3-GH-releasing peptide-6 or ghrelin receptor deletion abolishes the rapamycin-induced increment in food intake despite that plasma ghrelin remains elevated[5]. Together with the observation that mTOR is selectively expressed in a subpopulation of gastric endocrine cells and its activity is reciprocally related with the energy level, we propose that gastric mTOR is a peripheral fuel sensor integrating the energy supply with the food intake and energy metabolism by alteration of ghrelin production. Defining the mTOR signaling pathway to inhibit the production of acyl ghrelin, the active form of ghrelin, would shift therapeutic focus to gastric targets.

CONCLUSION

The fuel sensing mechanism in the central nervous system is critical for energy homeostasis. However, the anatomical structure and location of the hypothalamus pose significant hurdles for therapy targeting this organ. Searching for peripheral targets is appealing. Novel evidence suggests that mTOR is a critical regulatory molecule in gastric ghrelin cells and that its activity is linked to energy supply through modulation of the production of acyl ghrelin. Further studies will aim to advance our understanding of intracellular processes in the production of ghrelin and to provide new information on the integration of cellular activities of gastric endocrine cells with overall nutrient availability. Results of these new investigations will yield new insights relevant to treatment strategies for human obesity.

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REVIEW

Gene and cell therapy based treatment strategies for inflammatory bowel diseases

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Abstract

Inflammatory bowel diseases (IBD) are a group of chronic inflammatory disorders most commonly affecting young adults. Currently available therapies can result in induction and maintenance of remission, but are not curative and have sometimes important side effects. Advances in basic research in IBD have provided new therapeutic opportunities to target the inflammatory process involved. Gene and cell therapy approaches are suitable to prevent inflammation in the gastrointestinal tract and show therefore potential in the treatment of IBD. In this review, we present the current progress in the field of both gene and cell therapy and future prospects in the context of IBD. Regarding gene therapy, we focus on viral vectors and their applications in preclinical models. The focus for cell therapy is on regulatory T lymphocytes and mesenchymal stromal cells, their potential for the treatment of IBD and the progress made in both preclinical models and clinical trials.

Key words: Viral vector; Gene therapy; Cell therapy; Inflammatory bowel diseases; Immune tolerance; Regulatory T lymphocytes; Mesenchymal stromal cells

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases most commonly affecting young adults[1-3]. The exact pathogenesis is unknown, but it is widely accepted that IBD result from an inappropriate response of a defective mucosal immune system to the intestinal flora and other luminal antigens[4-6].

IBD include two major disorders: ulcerative colitis (UC) and Crohn's disease (CD). These disorders have distinct and overlapping pathologic and clinical characteristics[7]. UC is a relapsing non-transmural inflammatory condition that is limited to the colon[8]. Patients char-

acteristically present with bloody diarrhoea, passage of pus, mucus, or both, and abdominal cramping[8]. CD is a relapsing, transmural inflammatory disease of the gastrointestinal (GI) mucosa that can involve the entire GI tract from the mouth to the anus[8]. Patients characteristically present with discontinuous involvement of various portions of the GI tract and the development of complications including strictures, abscesses, or fistulas[8]. IBD are associated with a considerable reduction in quality of life of the patients[9-11] and currently no curative treatment options are available. Conventional therapeutics cannot prevent complications in IBD and although novel treatment strategies, including TNF-neutralizing antibodies, have greatly increased the therapeutic armamentarium, many patients still have to undergo surgery[12]. For this reason, the development of new treatments is required to prevent initiation of inflammation and, more importantly, allow for long-term remission. Gene and cell therapy approaches are more and more considered in relation to the prevention of inflammation in the GI tract. Gene therapy consists of the insertion or alteration of genes within an individual's cells to treat disease. Cell therapy describes the process of introducing new cells into a tissue in order to treat a disease. Both approaches have been applied successfully in a clinical setting for a broad range of diseases either separately or together, including early stage clinical development for IBD[13-23]. Here we discuss current progress in the field and future treatment prospects in the context of IBD.

GENE THERAPY AS TREATMENT FOR IBD

To facilitate the uptake and the expression of the transgene in the target cell, a vector is required. Vectors can be non-viral or viral. The choice of a safe and reliable vector that can mediate long-term gene transfer to both dividing and non-dividing cells is of vital importance for a gene therapy approach. Although viral vectors are created from pathogenic viruses, they are modified in such a way as to minimize their pathogenicity. This usually involves the deletion of a part of the viral genome critical for viral replication. Such a virus can efficiently infect cells and has the potential for long term stable gene expression. In the gene therapy section of this review we will focus on viral vectors that have been used successfully in gene therapy applications in recent years[14,15], are able to target the gut[24-32] and can therefore be considered for gene delivery in the GI tract, namely retro-, lenti-, adeno- and adeno associated viral vectors (for an overview see: Tables 1-3 or Figure 1).

For an overview of non-viral delivery methods to the intestine we recommend the review from O'Neill *et al*[33].

Retro- and lentiviral vectors

Retroviral vectors were used for the first time in a clinical setting over 20 years ago[34-36] and are among the most commonly used vectors in gene therapy. Retroviral particles require disruption of the nuclear membrane to gain access and therefore need cell division for entering the cell[37]. Retroviruses have been demonstrated to be able to transduce intestinal epithelial cells[24-26], although at a low efficiency. Alternatively, intestinal epithelial cells can be transduced efficiently by lentiviruses[27] which are a subclass of retroviruses. The lentiviruses have an advantage over retroviruses as vectors in gene therapy because of their ability to transduce non-dividing cells[38,39]. Furthermore the lentivirus did not induce mucosal damage or distribute beyond the distal colon[27] and appeared therefore as a potential vector for gene delivery in the treatment of IBD.

However, a safety issue to be considered with both retro- and lentiviral vectors is their potential to integrate at many sites in the human genome[40,41]. Those genomic integrations can result in insertional mutagenesis causing cancer development as has been observed in clinical trials[19,42-44]. Even though significant improvements in lentiviral vector safety have been achieved in recent years[45], the concern for random integration remains and needs to be addressed[46,47] before these vectors can be considered as safe tools for gene therapy applications in IBD.

Adenoviral vectors

Despite the fact that adenoviruses are pathogenic viruses and can cause morbidity, especially in immune-compromised patients[48], adenoviral vectors have been frequently used in gene therapy due to their broad tissue tropism and lack of integration into the host genome[49]. Gene therapy using adenoviral vectors has shown potential in the treatment of colitis in preclinical models[28-30]. For example, a single systemic injection of an adenoviral vector carrying the interleukin-10 (IL-10) transgene was sufficient not only to prevent the onset of colitis but also to induce clinical and histological remission in mice with established disease[29]. Additionally Schmiedlin-Ren *et al*[50] demonstrated that intestinal epithelial cells of IBD patients can be efficiently transduced *ex vivo* by adenoviral vectors. All together, these results suggest that targeting of the inflamed intestine through the luminal route can be possible using adenoviral vectors[50].

However, hematologic and hepatic toxicities were observed in animal studies after injection with high vector doses[51-53], which imply that further development in generating a new type of adenoviral vector is necessary before considering clinical applications. Recently a gutted adenovirus, devoid of all viral coding sequences, was shown to induce less toxicity[54] after delivery. However, this finding, if promising for future therapeutic applications, needs further exploration.

Adeno-associated virus vectors

The non-pathogenic, replication-deficient adeno-associated virus (AAV) holds promise for gene therapy. The AAV vector has a good safety profile as it remains predominantly episomal[55]. In general, 99% of recombinant AAV are maintained as episomal copies[56], indicating a

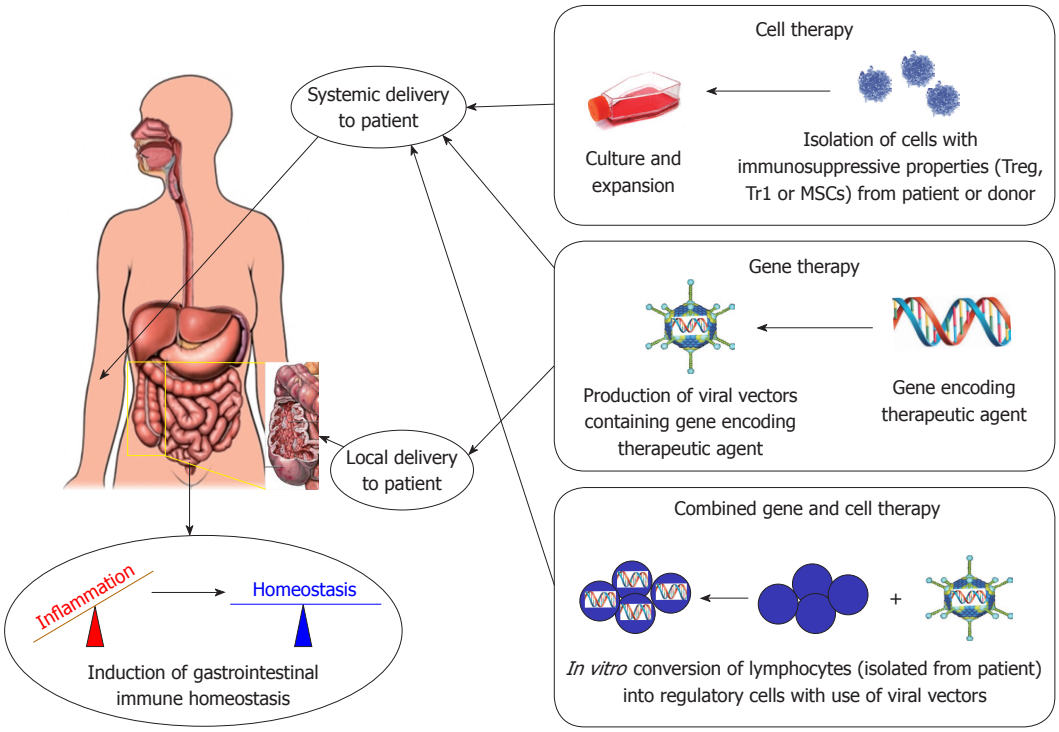

Figure 1 Emerging treatments for inflammatory bowel diseases. Overview of the gene and cell therapy based treatment strategies for inflammatory bowel diseases as discussed in this review. Treg: $CD4^+CD25^{high}FOXP3^+$ regulatory T cell; **Tr1: Type 1 regulatory T cell; MSC: Mesenchymal stromal cells.**

Table 1 Overview of the gene therapy based treatment strategies for inflammatory bowel diseases as discussed in this review

	Viral vector	Gut targeting	Reference for gut targeting	Status of development	Reference for status of development
Gene therapy	Retro- and lentiviral	Yes	[24-27]	Not performed	N/A
	Adenoviral	Yes	[28-30]	Preclinical	[28-30]
	AAV	Yes	[31,32]	Not performed	N/A

The status of development refers to research which has already been performed. AAV: Adeno-associated virus; N/A: Not applicable.

Table 2 Overview of the cell therapy based treatment strategies for inflammatory bowel diseases as discussed in this review

	Cell type	Generated from	Reference for generating cell type	Status of development	Reference for status of development
Cell therapy	Treg	Peripheral blood	[21,101,132-134]	Preclinical	[93,96]
	Tr1	Peripheral blood	[93,135]	Phase I clinical trial	(Unpublished data, UEGW 2010-ABS-577)
	Mesenchymal stromal cells	Adipose tissue/bone marrow	[17,22,23]	Phase I clinical trial	[17,22,23]

The status of development refers to research which has already been performed. Treg: $CD4^+CD25^{high}FOXP3^+$ regulatory T cell; Tr1: Type 1 regulatory T cell; UEGW: United European Gastroenterology Week.

Table 3 Overview of the combined gene and cell therapy based treatment strategies for inflammatory bowel diseases as discussed in this review

	Applied strategy	Viral vector used	Reference for viral vector used	Status of development	Reference for status of development
Combined gene and cell therapy	*Ex vivo* generated Treg/Tr1	Retrovirus	[113,115]	Preclinical	[113,115]
	T cell receptor transgenic Treg	Retrovirus	[116]	Preclinical	[116]

The status of development refers to research which has already been performed. Treg: $CD4^+CD25^{high}FOXP3^+$ regulatory T cell; Tr1: Type 1 regulatory T cell.

very low risk of insertional mutagenesis compared with retroviral vectors. Furthermore, AAV vectors are able to transduce both dividing and quiescent cells[57,58] and were demonstrated to be effective as gene therapy vectors in several promising preclinical models for autoimmune- and inflammatory disorders[59-66]. The therapeutic potential of the AAV as a vector in gene therapy has also been demonstrated in a clinical setting in recent studies[67-77].

AAV vectors were shown to be able to target the GI tract[31,32] and long term transgene expression post AAV treatment was reported which, in relation with the high turn-over of intestinal cells, suggests that transduction of the slow-dividing intestinal stem cells was achieved[31,32]. However, no data are presently available about the treatment of experimental colitis with AAV vectors.

CELL THERAPY AS TREATMENT FOR IBD

Cell-based therapies aim to introduce new cells into a tissue in order to treat a disease and can permit the replacement of function[78], or restore the homeostasis of the immune system[79]. In the last 50 years hematopoietic stem cell transplantation has been developed as a curative option for inherited disorders and hematologic or lymphoid cancers[13,80], leading the way toward innovative therapies for other illnesses. Recent results obtained from animal models and early human clinical trials in graft *versus* host disease but also CD showed that either regulatory T lymphocytes or mesenchymal stromal cells (MSCs) may be of clinical relevance for the treatment of IBD (for an overview see: Tables 1-3 or Figure 1).

Regulatory T lymphocytes

The immune system contains a population of T cells, called regulatory T lymphocytes that are specialized in immune suppression[81,82]. Low level autoimmunity may occur in the intestine as a result of the presence of the microbial flora or auto-reactive T cells. Regulatory T lymphocytes are generated in the mesenteric lymph nodes and subsequently migrate and expand in the gut[83], thereby preventing progress to chronic autoimmune disease[84,85]. These cells are able to suppress an immune response both by cell contact [e.g. killing or functional modulation of antigen presenting cells (APCs) or effector T cells] and soluble factor dependent mechanisms (e.g. secretion of immunosuppressive cytokines or deprivation of cytokines necessary for the expansion/survival of responder T cells)[86,87]. Antigen specific regulatory T lymphocytes have been described as having more therapeutic efficacy than polyclonal regulatory T cells[88-90]. In IBD the antigenic targets are not totally defined[6] and cell therapy would have to be restricted to polyclonal cells. However, regulatory T lymphocytes don't need to be antigen specific in order to suppress immune responses as a result of bystander suppression and infectious tolerance[91,92]. These are general mechanisms through which regulatory T lymphocytes are able to create a regulatory milieu *in vivo*[91,92] and could introduce tolerance in IBD.

Regulatory T lymphocytes were shown to be effective in both the cure and the prevention of experimental colitis in multiple animal models[93-96]. It was shown, for example, that transfer of regulatory T lymphocytes into mice with colitis led to resolution of the lamina propria infiltrate in the intestine and reappearance of normal intestinal architecture[96]. Therefore regulatory T lymphocytes could be used as a therapeutic tool in IBD where their homeostasis is disturbed[97,98].

Among the different T cells with suppressive activity the $CD4^+CD25^{high}FOXP3^+$ regulatory T cell (Treg)[82] and the type 1 regulatory T cell (Tr1)[81] subsets are the most well-defined so far. The Tr1 is typically characterized based on the cytokine production profile ($IL\text{-}10^{high}$)[81] and Treg by the expression of the transcription factor Forkhead box p3 (FOXP3 in humans/Foxp3 in mice), which appears to function as the master regulator in their development and function[99,100].

Treg and Tr1 have the potential to prevent or cure colitis[93-96] and a favourable safety profile was demonstrated in phase I clinical trials[21,101]. Tr1 were shown to have a preliminary efficacy signal in patients in a phase I clinical trial for refractory CD (unpublished data, UEGW 2010-ABS-577). Currently the efficacy of Treg and Tr1 based cell therapy awaits further confirmation from phase Ⅱ/Ⅲ clinical trials but overall these results emphasize that both Treg and Tr1 are promising tools for therapeutic applications in IBD.

MSCs

MSCs are non-haematopoietic stromal cells exhibiting multi-lineage differentiation capacity and the ability to mediate immunosuppressive and anti-inflammatory effects[102-105]. The exact mechanism by which MSCs suppress the immune system is not fully understood. It is known, however, that MSCs have immunosuppressive features in common with regulatory T lymphocytes, as for example preventing the maturation of APCs[102] or physically hindering T cells from contacting APCs[103]. Additionally, it was shown that FOXP3 expression confers a greater immunosuppressive potential to MSCs[106].

MSCs can be isolated from various tissues[107-109] and were shown to ameliorate experimental colitis[110,111]. In humans, MSCs obtained from adipose tissue induced healing in perianal fistulas in patients with CD[17]. Furthermore, in a phase I clinical trial, administration of autologous bone marrow-derived MSCs was shown to be safe and feasible in the treatment of refractory CD[22]. Additionally it was demonstrated that *ex vivo* expanded autologous bone marrow-derived MSCs are a safe and feasible approach for intrafistular injections in patients with CD[23]. These results[17,22,23] show potential and await further verification in phase Ⅱ/Ⅲ clinical trials which are currently being conducted.

CAN GENE AND CELL THERAPY OVERLAP IN THE TREATMENT OF IBD?

The phenotype and function of lymphocytes can be

modified using viral vectors, to create tools for a cell therapy approach in the treatment of autoimmune-, and inflammatory disorders[112] and by consequent IBD[113-116]. It was shown that the *ex vivo* targeting of spleen derived $CD4^+$ T cells by a retroviral vector expressing IL-10 was able to generate Tr1 that prevented colitis in an experimental model of IBD[113].

By the same approach, fully functional Treg were generated by transduction of T cells with a Foxp3 transgene. These cells were able to suppress autoimmunity and graft rejection *in vivo*[89,115,117,118]. Furthermore, Hori *et al*[115] showed that the *in vitro* generated Treg prevented colitis in a mice model of IBD.

Additionally it was demonstrated that Treg can be efficiently transduced to express functional antigen-specific receptors[116]. Adoptive transfer of small numbers of these transduced Treg was associated with antigen-specific, dose-dependent amelioration of experimental colitis in mice[116].

GENERAL CONSIDERATIONS RELEVANT FOR IBD

The route of therapeutic delivery is important when considering gene or cell therapy in relation with IBD. The mucus lining in the intestine is a barrier for gene transfer *via* the luminal route[119] and the clearance of viral particles by the liver represents a problem for the systemic delivery[120]. Nonetheless, as described above it has been shown that transduction *via* these routes is possible and that long term transgene expression can be achieved. Possible viral vectors, as for example the AAV based viral vectors seem to have the potential to transduce the GI tract, but the optimization of gene targeting to the gut needs to be further explored. This could be achieved by testing different AAV serotypes[121] or modifying the AAV capsid[122]. A promising method is the socalled DNA shuffling method. DNA shuffling is a method whereby genes are rearranged to form hybrid genes with new properties[123]. This can be done using polymerase chain reactions, as described by Cohen[123]. If this approach is used for genes encoding AAV capsid proteins it can allow for the development of cell type specific vectors[124] and thereby shows promise for creating a gut targeting AAV. Furthermore, chemical redirection of the AAV capsid shows potential in engineering vectors with novel tissue tropisms[125]. Chemical engineering refers to a process whereby the amino acids on the surface of the AAV capsid are changed[125]. This method has proved to be successful in redirecting the AAV from liver to skeletal and cardiac muscle following systemic administration in mice[125] and could therefore have potential in directing the AAV to the GI tract.

Due to the presence of stem cells in intestinal crypts[126] the gut is suggested to be an interesting target for therapeutic gene transfer. Every crypt in the intestine contains four to six independent stem cells[126]. Stem cells are believed to divide very rarely[126]. Therefore, these cells could have the potential to permit long term, stable transgene expression after transduction. It has been shown that intestinal stem cells can be transduced *in vitro* using a retroviral vector[127]. Long term transgene expression observed in the gut after AAV vector delivery in mice suggests that transduction of intestinal stem cells is possible *in vivo*[31,32].

FUTURE PROSPECTS

Knowledge of the pathophysiology of IBD is growing and it has become clear that significant genetic as well as phenotypic heterogenecity exists within both CD and UC[128]. These findings offer opportunities for more specifically targeted interventions. Gene or cell therapy based treatment strategies can be adapted and targeted exclusively at certain subgroups within the IBD patient population with characterized genetic defects linked to the impairment of their gut physiology.

Strategies to optimize gene therapy approaches include the use of a tissue specific promoter enabling site specific expression of a transgene. Recently, gut specific promoters have been described[129-131]. The A33-antigen promoter for example strictly depends on the presence of the intestine-specific transcription factor Cdx1 which is essential for the unique intestinal expression pattern of the A33-antigen gene[129,131]. Therefore this promoter is a promising candidate to induce intestine specific expression of a transgene[131].

CONCLUSION

IBD are a group of chronic inflammatory disorders most commonly affecting young adults and currently there is no curative treatment available. A gene therapy approach for the local expression of therapeutic agents in the gut or a cell therapy approach using regulatory T cells or MSCs may offer an alternative treatment for GI inflammation. Both gene and cell therapy approaches have shown promising results in preclinical models of IBD. Cell therapy approaches have been translated to a clinical setting and currently phase Ⅱ/Ⅲ clinical trials for the treatment of refractory CD are in progress. Concerning gene therapy, further development of viral vector delivery to the gut as well as long term efficacy are still needed, but pre-clinical data are promising.

Overall, both gene and cell therapy have the potential to become important players in the next generation of therapeutic agents that will be aimed at unmet medical needs such as those that exist in IBD.

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REVIEW

Enterocytes' tight junctions: From molecules to diseases

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Abstract

Tight junctions (TJs) are structures between cells where cells appear in the closest possible contact. They are responsible for sealing compartments when epithelial sheets are generated. They regulate the permeability of ions, (macro) molecules and cells *via* the paracellular pathway. Their structure at the electron microscopic level has been well known since the 1970s; however, only recently has their macromolecular composition been revealed. This review first examines the major macromolecular components of the TJs (occludin, claudins, junctional adhesion molecule and tricellulin) and then the associated macromolecules at the intracellular plaque [zonula occludens (ZO)-1, ZO-2, ZO-3, AF-6, cingulin, 7H6]. Emphasis is given to their interactions in order to begin to understand the mode of assembly of TJs. The functional significance of TJs is detailed and several mechanisms and factors involved are discussed briefly. Emphasis is given to the role of intestinal TJs and the alterations observed or speculated in diverse disease states. Specifically, intestinal TJs may exert a pathogenetic role in intestinal (inflammatory bowel disease, celiac disease) and extraintestinal diseases (diabetes type 1, food allergies, autoimmune diseases). Additionally, intestinal TJs may be secondarily disrupted during the course of diverse diseases, subsequently allowing the bacterial translocation phenomenon and promoting the systemic inflammatory response, which is often associated with clinical deterioration. The major questions in the field are highlighted.

Key words: Tight junctions; Occludin; Claudins; Junctional adhesion molecule; Tricellulin; Intestinal permeability

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INTRODUCTION

The direct observation of how close cells can be came with the use of the transmission electron microscope. Cell biologists of the 1950s and 1960s observed several structures that seemed to bring the cells into close contact and connect with each other, and gave to these junctions several names, descriptive of either the structure or the function served by the structure. Thus, the names of occluding junctions [or tight junctions (TJs)], anchoring junctions (or adherens junctions) and communicating junctions (or gap junctions) were coined.

TJs are the key elements for creating two different barriers: the first barrier is between the apical and basolateral cell membrane (lipid bilayer) compartment, thus keeping the protein and lipid composition of these two membrane domains qualitatively different by restricting

their exchange. The second barrier is between the apical and basal compartment defined by the epithelium in which the TJ is present, thus restricting the passage of water, solutes and cells from the "outer" to the "inner" compartment and vice versa, also known as paracellular pathway.

TJs were seen in the most apical part of the lateral cell membrane of polarized cells, forming continuous circumferential contacts. What morphologists observed with the use of transmission electron microscopy was an obliteration of the extracellular space between cells at certain points; the morphological data led them to suggest that there are points of the closest possible apposition between the cell membranes of cells involved. The use of freeze-fracture techniques added important new information by showing these "contact points' as fibrillar rows of intramembrane particles, forming a branching network around the cell. The chemical composition of these particles became a subject of intense investigation. It was originally proposed that these particles are composed solely of lipids[1]. Starting from the early 1990s, proteins localized at and involved in the formation of TJs have been identified. The first member of this family, occludin, is described in the report by Furuse *et al*[2] in 1993 and since then the field has witnessed a wealth of new information, which will be briefly reviewed in this report.

MACROMOLECULAR COMPOSITION OF TJs

To date, four groups of macromolecules are considered as bona fide integral components of the TJ: occludin(s), claudins, junctional adhesion molecule (JAM) and tricellulin. It is remarkable that the members of the first two groups cross the cell membrane four times, thus creating two extracellular loops and three intracellular domains, including the amino terminal domain, the carboxy terminal domain and the very short loop-connecting segment. In contrast, JAM crosses the cell membrane only once (Figures 1-3). Recently, tricellulin was identified as another integral TJ protein, quite homologous to occludin, with 4 transmembrane domains, preferentially localized at the tight junctional strands of tricellular contacts of epithelial cells.

Occludin

The name comes from the Latin verb "occludere", which means to restrict passage. It was first isolated by Furuse *et al*[2] in 1993 in cultures of chicken liver cells, based on the results of intensive screening of monoclonal antibodies. It is a component of TJs, highly conserved among species and without tissue specific isoforms (recently only one occludin isoforms resulting from alternative splicing has been reported)[3]. Occludin is only expressed under normal conditions in cells that form TJs. When cells that do not form TJs are led *via* transfection to express occludin, it is concentrated at cell contact sites, forming only a small number of short strands identified in freeze-fracture replicas[4]. However, when occludin was cotransfected with claudin-1, tight junctional fibrils were formed[4]. In addition, immunoreplica electron microscopy has documented the presence of occludin in tight junctional strands[5].

Occludin (Figure 1) consists of 504 amino acids. The amino terminal portion is intracellular and contains 57 amino acids. This portion contains a WW domain (PPYP), which is motif participating in the interaction with other signaling and regulatory proteins[6]. The four transmembrane domains consist of 21-24 amino acid residues, mostly hydrophobic. The two extracellular loops are 43 amino acids long each and the intravellular loop is 10 amino acids long. Both extracellular loops are rich in tyrosine and glycine (in the range of 20%-30%); however, the functional significance of this is not known yet[2]. The very long intracellular carboxyl terminal part (50% of the sequence) has several sites of potential interaction with other macromolecules and phosphorylation sites[2,7]. It is a highly hydrophilic sequence, containing stretches of charged amino acids, like EEEEE (aa 347-351) and RRGRRRRR (aa 363-370).

The electrophoretic mobility of occludin in polyacrylamide gels has revealed a wide range of bands from 62kDa to 82 kDa. This difference in mobility has been assigned to post-translational modifications and more precisely to differential phosphorylation on both tyrosine and serine/threonine residues. It has been proposed that the site and the degree of phosphorylation of occludin are important parameters in defining the localization and the function of the molecule. For example, highly phosphorylated occludin, especially in serine/threonine residues, is detected mainly in the junctional area and correlates with restricted transcellular permeability. On the contrary, non-phosphorylated occludin is detected intracellularly, in the vesicular compartment, and is considered to contribute less to transcellular permeability, being a pool of macromolecules easily subjected to regulatory stimuli. As mentioned above, the site of phosphorylation is also important. It has been suggested that high degree of phosphorylation at tyrosine residues correlates with loss of function and higher transcellular permeability.

Recently, a novel alternatively spliced form of occludin has been described[3], in which 56 amino acids are present close to the amino terminal of the molecule. This new form termed occludin 1B has been detected in TJs and widely co-expressed with the known occludin.

Claudins

The name claudin comes from the Latin verb "claudere", which means "to close". Claudins, identified after occludin, are transmembrane proteins extremely crucial for TJ formation. The existence of another TJ component, in addition to occludin, was suspected when Saitou *et al*[8] created an occludin deficient cell line that was surprisingly able to form TJ strands. These TJs appeared structurally normal, but this finding should not underestimate the role of occludin since a later studied model of occludin defi-

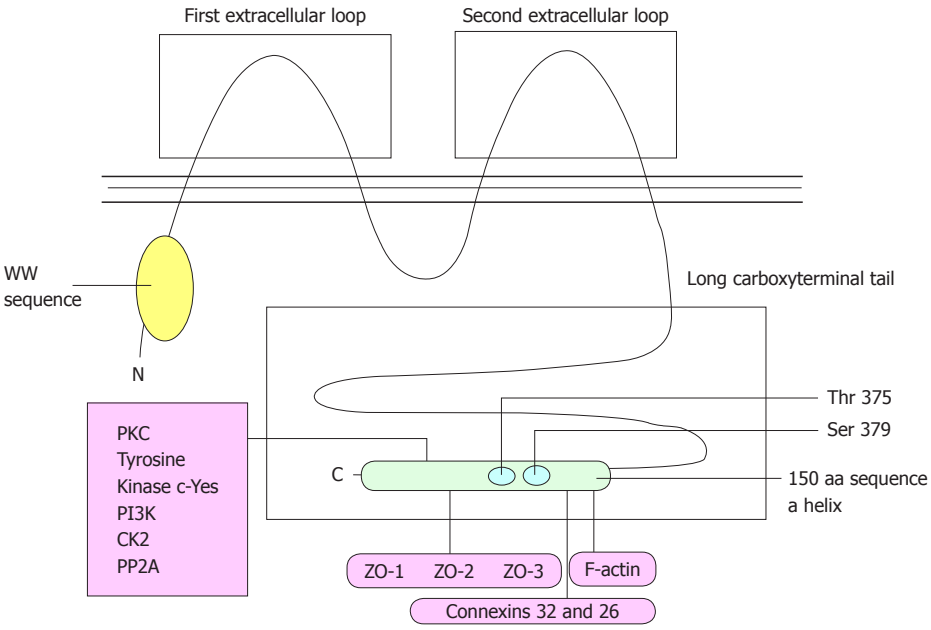

Figure 1 Occludin. WW sequences are highly preserved 35-45 aminoacid segments, containing Trp and Pro. They recognize proline rich domains. Ser 379 and Thr 375 are casein kinase 2 (CK2) phosphorylation sites. PKC: Protein kinase C; PI3K: Phosphatidylinositol 3 kinase; PP2A: Protein phosphatase 2A; ZO: Zonula occludens.

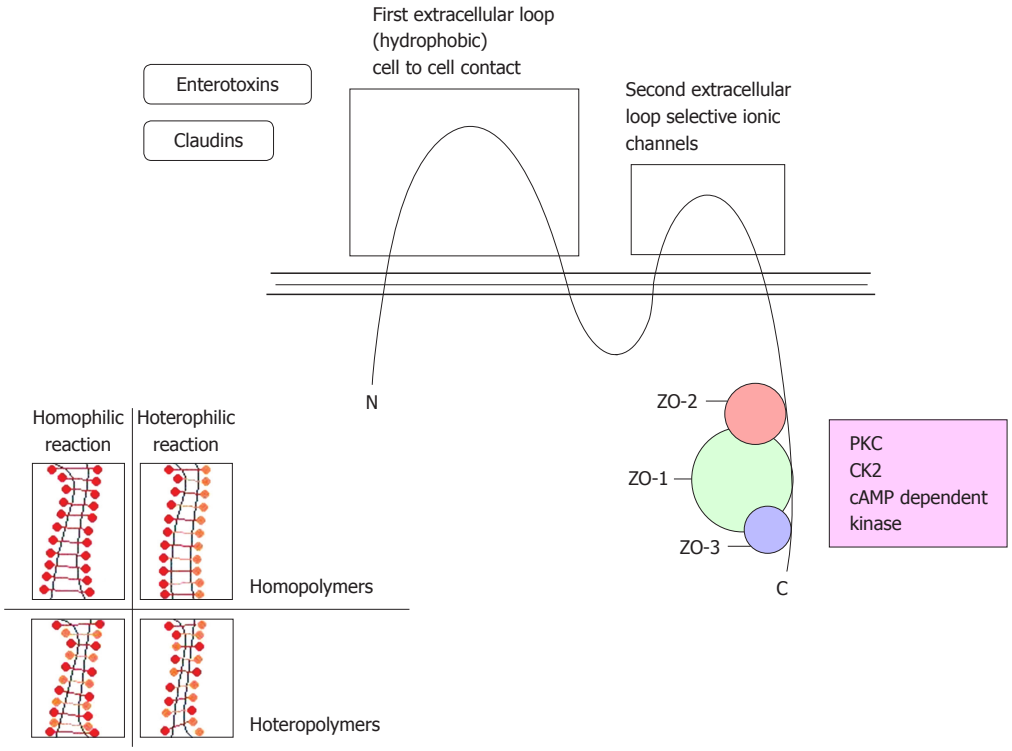

Figure 2 Claudin(s). PKC: Protein kinase C; CK2: Casein kinase 2; ZO: Zonula occludens.

cient mice[9] presented abnormalities from several tissues e.g., chronic inflammation and hyperplasia of the gastric epithelium, calcification in the brain, testicular atrophy, thinning of the compact bone and loss of cytoplasmic granules in striated duct cells of the salivary glands.

Furuse *et al*[10] identified the first two members of the claudin superfamily, claudins 1 and 2. Comparing their sequence to already known proteins, they found similarity to at least three membrane-associated proteins: rat ventral prostate-1, clostridium perfringens enterotoxin receptor and oligodendrocyte specific protein[11]. Up until now, the claudin family consists of 24 members in humans[12,13] (Table 1).

Claudins are transmembrane proteins with MW rang-

Table 1 Distinctive characteristics of claudins

Claudin	Distinctive characteristics
1	Present in high resistance epithelia (collecting segment), absent in leaky epithelia (proximal tubule)
	Crucial for the mammalian epidermal barrier
	Mutations cause neonatal sclerosing cholangitis
	Prognostic value in colon and thyroid cancer
2	Present in leaky epithelia (proximal tubule) and absent in tight epithelia
	Present in the choroids plexus epithelium
3	Present in the tighter segments of the nephron
	Up-regulated in ovarian, breast, prostate and pancreatic tumors
4	Induces selective decrease in sodium permeability
	Present in the tighter segments of the nephron
	Alternative name: CPE-R
	Up-regulated in ovarian, breast, prostate and pancreatic tumors
5	Frequently deleted in velo cardio facial syndrome
	Constitutes TJ strands in endothelial cells and it is transiently expressed during the development of retinal pigment epithelium
6	Present in embryonic epithelia
	Its overexpression in transgenic mice generates a defective epidermal permeability barrier
7	Down regulated in head and neck squamous cell carcinomas
	Upregulated in stomach cancer
8	Present in the tighter segments of the nephron
10	Prognostic value in hepatocellular carcinoma (recurrence)
11	Present in oligodendrocytes and sertoli cells; also named OSP
14	Expressed in the sensory epithelium of the organ of Corti Mutations cause autosomal recessive deafness
15	Present in endothelial cells
16	Critical for Mg^{2+} and Ca^{2+} resorption in the human thick ascending limb of Henle
	Mutations cause familial hypomagnesaemia
18	Expressed in the lung and stomach

Claudins 9, 12, 13, 17, 19-24: Insufficient data. CPE-R: Clostridium perfringens enterotoxin receptor; OSP: Oligodendrocyte sertoli protein.

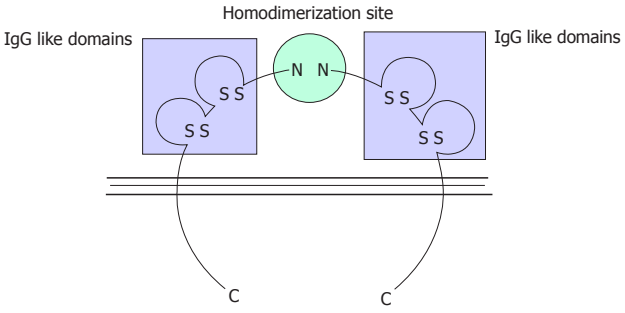

Figure 3 Junctional adhesion molecule.

ing from 20 kDa to 27 kDa. They possess an intracellular amino-terminus, four transmembrane segments that form two extracellular and an intracellular loop, and a short carboxyterminal intramembrane tail (Figure 2)[14]. The first and fourth transmembrane part, as well as the first and second extracellular loops are highly conserved, whereas the COOH tail has the greatest variability among members of the claudin family[11]. The first extracellular loop is longer and more hydrophobic than the second one[11,14]; therefore it is possibly responsible for cell-to-cell contact. The second extracellular loop contains many charged residues, which regulate the affinity to ions[14] and possibly forms ion-selective paracellular channels. The carboxyterminal tail contains many phosphorylation sites, related to protein kinase C (PKC), casein kinase 2 (CK2) and a cAMP dependent kinase. Almost every claudin has a carboxyterminal Tyr-Val sequence, which acts as a PDZ-domain interacting motif. Exceptions are claudins 16, 11, 12 and 13. When claudins 1 and 2 were transfected in TJs, negative cells[4] induced cell-to-cell adhesion by forming TJ strands with no obvious structural and functional difference from their occludin positive homologues. At present, claudins are supposed to be the "backbone" of the TJ barrier[14-16].

TJs *in vivo* usually express 2 claudins[15] with two notable exceptions, the oligodendrocytes and the Sertoli cells, both expressing a single claudin, claudin-11[11]. Claudins are also characterized by a differential tissue expression pattern[15,17] and a developmental selectivity. Interestingly, nephron expresses different claudins in different segments and this variability may be the basis for differential epithelial permeability in these segments[18-20]: claudins 5 and 15 are expressed at endothelial cells, 2, 10 and 11 at the proximal tubule segment, 1, 3 and 8 at the distal tubule and 1, 3, 4 and 8 at the collecting duct segment. As proved by Turksen *et al*[21,22] and Reyes *et al*[18], the expression of claudins in murine nephron changes during development. Hellani *et al*[23], who studied the expression of claudin 11 on Sertoli cells, also verified the above observations.

The complexity at the expression of different claudins in different tissues, in different stages of their development and to more than one combination, suggests that these molecules participate in both homophilic and heterophilic interactions within the same cell or between opposing cell membranes[24]. The possible combination models that claudins follow to their polymerization are

presented in Figure 2. At the level of electron microscopy, claudins form different strand patterns, related to the P face or the E face of the cytoplasmic membrane. Claudins 1, 3 and 11 form P face strands, whereas claudins 2 and 5 are related to the E face strand pattern[15,24-26].

Claudins form a transmembrane and transcellular net that serves paracellular permeability, epithelial polarization and conservation of the transepithelial resistance (TER)[11,14,16], as well as selective permeation of charged molecules and ions[27,28]. These parameters vary among different tissues according to their claudin expression, as long as claudins are the only known variable elements in TJs[11].

Finally, some members of the claudin family are receptors for extracellular ligands, such as Clostridium perfringens enterotoxin, that bind directly to claudins-4 (high affinity) and -3 (low affinity)[29-31].

JAM

JAM was first described in 1998 by Martìn-Padura *et al*[32]. It is a type I transmembrane protein of MW 43 kDa, belongs to the immunoglobulin superfamily (Figure 3) and is localized in close proximity to the TJs of epithelial and endothelial sheets.

When JAM is expressed in cells that do not form TJs under physiological conditions, tight junctional strands are not observed, as is the case with occludin and claudins[33]. In this case, JAM molecules are accumulated only when cell-cell contact is established and both cells express JAM[32], suggesting a specific polymerization pattern by homophilic interactions. It appears that JAM plays a role in the process of formation of TJs, since antibodies against JAM do not disrupt already formed TJs[34].

Structurally, JAM consists of an extracellular amino terminal segment, a transmembrane domain and an intracellular carboxy terminal segment[32]. The extracellular part consists of 215 amino acids and contains two variable (V) type IgG domains. The V-V arrangement is novel between Ig domains and differentiates JAM-subfamily from other subfamilies of the Ig superfamily[32]. The intracellular part consists of 45 amino acids, where motifs appropriate for interactions with occludin[32] as well as other TJ associated macromolecules exist. The recently revealed X-ray structure of JAM suggests that first a U-shaped homodimer is formed and several homodimers interact and form an extensive network[35].

JAMs are subdivided based on the expression of type Ⅰ or Ⅱ PDZ-binding motifs in the intracellular C-terminus, which suggests that the 2 types interact with unique scaffolding and cytoplasmic proteins. JAM-A, JAM-B, and JAM-C (or JAM-1, JAM-2, and JAM-3) have type Ⅱ binding motifs, whereas the atypical JAMs, including JAM-4, Coxsackie and adenovirus receptor and endothelial selective adhesion molecule, contain type Ⅰ PDZ-binding domains[36].

It has been proposed that JAM may contribute to free diffusion of proteins within the lipid bilayer but more importantly to the restriction of cellular passage (leukocytes, monocytes, lymphocytes, *etc*.) through TJs between endothelial cells[14,32,35,37].

Tricellulin

A recent addition to the list of TJ proteins is tricellulin. Structurally it is quite homologous to occludin, with 555 amino acids forming 4 transmembrane domains; both the amino and the carboxy termini are intracellular, forming two extracellular loops. It is localized at the tight junctional strands of tricellular contacts of epithelial cells[38]. It participates in epithelial barrier organization. It was recently found that recessive mutations in the tricellulin gene cause nonsyndromic deafness and that tricellulin participates in the junctions in cochlear and vestibular epithelial cells[39]. Recent studies indicate that tricellulin is also localized in special TJs of myelinating Schwann cells[40]. Tricellulin has also been proposed to play an inhibitory role in the process of epithelial to mesenchymal transition of gastric carcinoma cells[41].

MACROMOLECULAR COMPONENTS ASSOCIATED WITH TJs

Several macromolecules are concentrated underneath the TJs and constitute the "tight junctional plaque". In general, their function is to promote interactions between them and the bona fide TJ macromolecules described above. These interactions can be mediated through: (1) PDZ motifs, that are present in several copies in most of the proteins of the tight junctional plaque; these motifs are usually 80-90 amino acids long and associate with certain protein motifs (e.g., S/TXV). Their presence in high numbers allows multiple and complicated interactions, leading to clustering and anchoring of transmembrane proteins[42]; (2) SH3 motifs, usually 50-70 amino acids long and frequently used as docking sites for several intracellular proteins; and (3) guanylate kinase (GK) motifs that are considered to be enzymatically inactive but could activate signaling pathways[43]. The proteins that contain them are collectively termed MAGUK proteins (Membrane-associated GK proteins). These proteins are beyond the scope of the present review so they will only be briefly mentioned, with emphasis on their interactions.

Zonula occludens-1

Zonula occludens-1 (ZO-1) is a 210-225 kDa phosphoprotein that interacts with occludin[44], claudins[45] and JAM[46] and also ZO-2[47], ZO-3[47], AF-6[46], cingulin[48] and the actin cytoskeleton[44]. Therefore, it plays a key role in bringing several components together and in connecting tight junctional proteins to the cytoskeleton. Cells that do not form TJs contain ZO-1 either dispersed in the cytoplasm or concentrated in cadherin-enriched adherens junctions[49] (Table 2).

ZO-2

ZO-2 is a 160 kDa protein present in two different isoforms. Table 3 shows the relationships of ZO-2 to other TJ molecules and cytosceleton fibers[14]. ZO-2 reacts with the splicing factor SC35[50] and with the transcription fac-

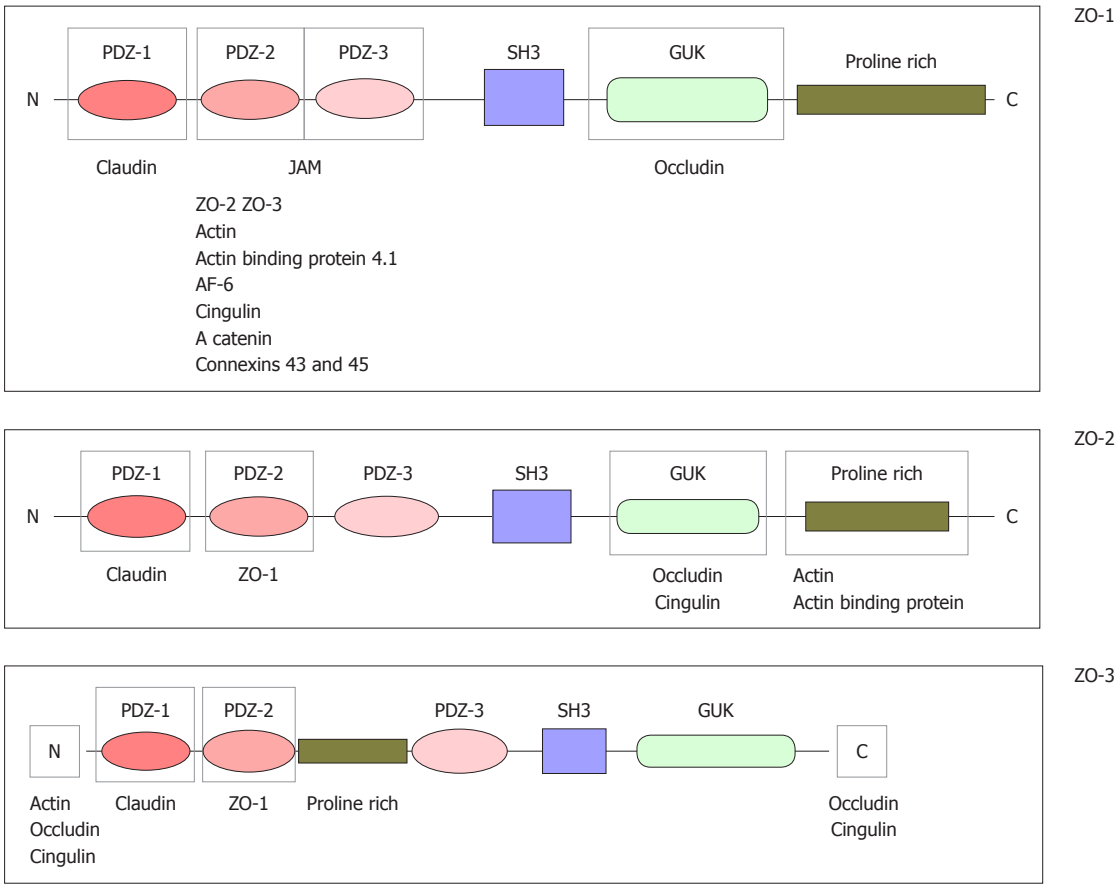

Figure 4 Zonula occludens. JAM: Junctional adhesion molecule; ZO: Zonula occludens.

Table 2 Interaction between zonula occludens-1 and other macromolecules

ZO-1 protein domain	Interacting molecules
1st PDZ	C terminus of claudin
2nd/3rd PDZ	JAM
GUK	Occludin
	CAR
2nd PDZ	ZO-2s and ZO-3s 2nd PDZ
	Actin cytoskeleton
	Actin binding protein 4.1
	AF-6
	Cingulin
	A catenin
	Connexins 43 and 45

ZO: Zonula occludens; CAR: Coxsackievirus and adenovirus receptor; JAM: Junction adhesion molecule.

tors Fos, Jun and C/EBP[51].

ZO-3

ZO-3 is a 130 kDa that differs from the other two members of ZO group in that it lacks the long carboxy-tail (Table 4). According to the model of Wittchen *et al*[47], ZO-1 binds to ZO-2 and ZO-3; however, there is no direct interaction between ZO-2 and ZO-3 (Figure 4).

Table 3 Interaction between zonula occludens-2 and other macromolecules

ZO-2 binding area	Interacting molecule
1st PDZ	Claudin
2nd PDZ	ZO-1
GUK	Occludin
	Cingulin
C terminal proline rich domain	Actin
	Actin binding protein 4.1

ZO: Zonula occludens.

AF-6/Afadin

AF-6/Afadin is a 205 kDa protein that seems to be more important for TJ formation and development than for TJ stabilization[14]. It is expressed in both TJ and AJ, and it seems to correlate with: (1) ZO-1 and activated Ras protein have antagonistic effect for the same binding site[52]; (2) JAM; and (3) F actin. AF-6 also interacts with cingulin[48]. Afadin expresses a 190 kDa splicing variant[53], found at the postsynaptic densities of neuronal tissues.

Cingulin

Cingulin is named after the Latin word "cingere", to encircle. It is a 140-160 kDa protein. It is characterized

Table 4 Interaction between zonula occludens-3 and other macromolecules

ZO-3 binding sites	Interacting molecules	
1st PDZ domain	Claudins	
2nd PDZ domain	ZO-1	
N terminus	Actin	Occludin
C terminus (class I PDZ binding motif TDL)	6th PDZ domain PATJ	Cingulin
	Connexin 45	

ZO: Zonula occludens.

by a globular N terminal head, a central α-helical coiled-coil domain and a C terminal tail. Through its central rods, dimmers are formed that interact with ZO-1, ZO-2, ZO-3, JAM, F actin and myosin. The majority of these interactions are supported by its globular head[14]. Cingulin is a serine phosphorylated protein, independent from PKC[54].

7H6 antigen

One hundred and fifty five kilodaltons protein that belongs to a family of proteins with alpha helical coiled coil domain and an ATPase domain. It sustains phosphorylation and its phosphorylated form is detected at TJ areas, whereas the non-phosphorylated form dissociates form TJs[14].

ASSEMBLY OF TJs

During embryogenesis and formation of epithelial sheets, it appears that primordial "spot-junctions" are formed. These are found in protrusions of neighboring cells and consist of cadherin, nectin, AF-6 and ZO-1[55,56]. At a later stage, cadherin and nectin are removed from those spots destined to become TJs, while occludin and claudins are recruited[57]. AF-6 remains in TJs of endothelial sheets but is removed from mature epithelial sheets and JAM is detected only in its presence in the tight junctional area[46], strongly suggesting a co-operative role of JAM and AF-6 related to specific cellular transmigration.

The interactions between the macromolecules described above obviously play an important role in the assembly process. Although several facts of the assembly process are not known yet, one can highlight observations that are the first important clues we have for understanding this process. Phosphorylation and dephosphorylation of occludin is a crucial post-translational modification since it has been connected with assignment of occludin to different subcellular compartments. Kinases and phosphatases involved are expected to be major players in the assembly-disassembly process. The intracellular domain of occludin is key to these events, since it contains several motifs that allow binding to the other macromolecules (see above). The last 150 amino acids close to the carboxyl terminal are very highly conserved among species and it is believed that they contain the self-association domain, critical for fibril formation.

Freeze-fracture immunostaining data suggest that usually two different types of claudins are present in tight junctional strands. This finding suggests that specific areas belonging to the short intracellular domains of claudins are responsible for crucial interactions between claudins and between one claudin and one occludin molecule. Although the molecular basis for these interactions is not known yet, it is certain that they will prove of great importance in understanding how the differential distribution of claudins in tissues leads to the great variety of electrical resistance and permeability observed among epithelial and endothelial sheets.

Another important aspect for our understanding of TJ assembly and functionality is the precise nature of the associations between extracellular loops of occludin and claudins. The length of each loop, similar in occludin and very different in claudins and the specific amino acid composition, mentioned above, are likely to be major factors in the formation of the barrier (Figure 5).

FUNCTIONAL SIGNIFICANCE OF TJs-REGULATORY MECHANISMS

TJs are crucial structures for the establishment and the stability of epithelial barriers. They mediate cell to cell adhesion, thus creating a mechanical and charged fence for selective permeability of macromolecules and ions. Ionic permeability is expressed through the parameter of TER. Macromolecular and ionic permeability seems to be quite independent, as they sustain controversial remodeling under the same conditions[58].

More than a simple paracellular fence, the transmembrane component of TJs blocks the circulation of proteins and lipids between the apical and basolateral membrane, mediating membrane polarization. Finally, the intracellular component triggers a variety of signaling pathways and communicates with the nucleus, acting as a "sensor" for extracellular events.

TJ transmembrane and intracellular proteins interact to cytoskeletal elements and undergo various types of phosphorylation. They also regulate protein expression, participating in vesicle trafficking. Pit and Rab proteins are the intracellular proteins that mediate such a function[14]. Finally, TJs interact with the actin cytoskeleton, and directly or indirectly are related to other membrane structures and macromolecules, like adherens junctions[14,16], gap junctions and proteoglycans[14].

The extracellular part of the TJs, consisting of the extracellular part of transmembrane proteins, acts as a multidynamic ligand for both homophilic and heterophilic interactions. It interacts with other cells participating with homodimers (occludin) and homopolymers (JAM) of heteropolymers (claudins). Additionally, free antigens from the extracellular space are ligands for extracellular TJ domains. Between these antigens are bacterial toxins, whose role in the pathogenetic mechanisms is discussed below.

TJs are in a dynamic balance, regulated both from intracellular and extracellular events. Intracellular events

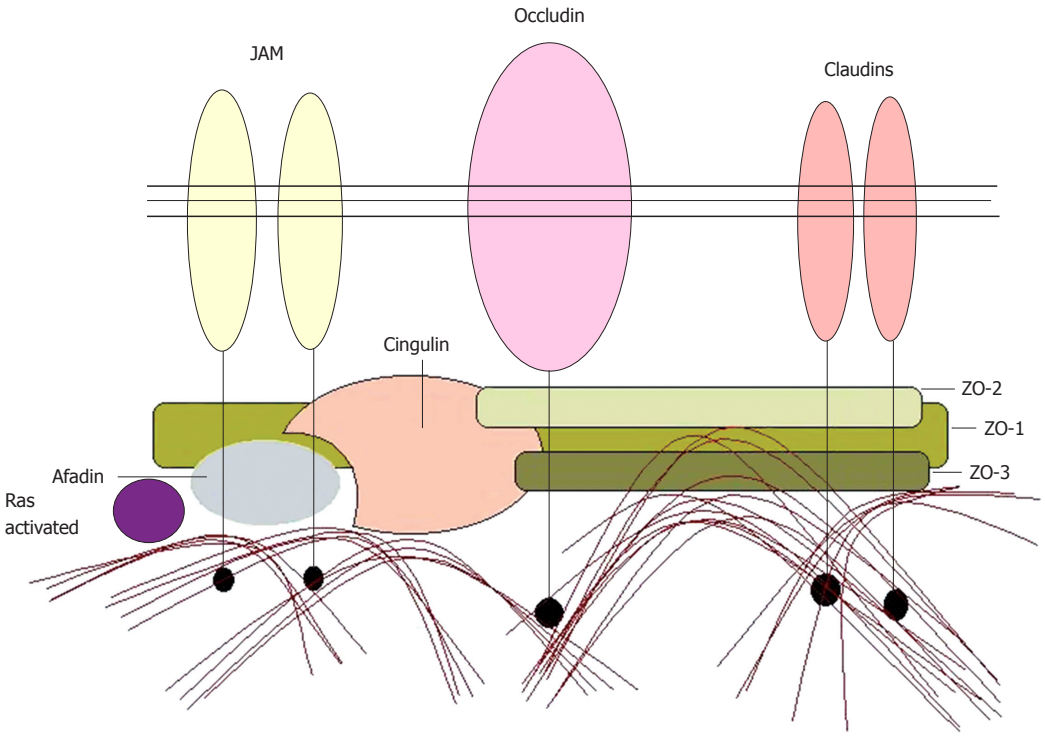

Figure 5 Assembly of tight junction proteins. JAM: Junctional adhesion molecule; ZO: Zonula occludens.

that may influence TJ stability are related to energy depletion and cAMP level changes. ATP depletion downregulates TJs[59], whereas cAMP induces increase of TER and reduced paracellular permeability[58]. Cell cycle also affects TJs morphology, according to development of differentiation of the cell. Cell cycle regulation on TJs can be both quantitative (enhanced or reduced expression of the same molecular substates) and qualitative (changes of molecular substrates, e.g., differential expression of claudins during development). Extracellular events with the potential for TJ regulation include the following five aspects. Direct interaction of TJ proteins to other cell proteins; a typical example is endothelial interaction to leukocyte membrane antigens that induces site-specific TJ dissociation[60]; direct interactions to external antigens: claudins 3 and 4 and occludin are both receptors for Clostridium perfringens enterotoxin[29,61,62]; indirect paracellular (cytokine) effects and hormonic stimulus: (1) proteases[63] - leukocytes are proposed to secrete proteases that destroy TJs by macromolecular cleavage; (2) interleukins[60,64,65] - interleukins can have enhancing or dissociative effect on TJs, depending on the type of interleukin acting and the tissue studied; (3) interferons[60,64,66] - like interleukins, interferons can have both up or downregulating effect on different epithelia; (4) Igs[67] - IgM and IgG interact with coxsackievirus and adenovirus receptor (CAR) at inflammation sites, which may play a role at neutrophil transmigration; (5) GFs[58,68,69] - growth factors generally act on epithelia by lysis of their barrier, thereby dissociation of TJs. Both fibroblast growth factor, hepatocyte growth factor/scattering factor and vascular endothelial growth factor decreased TER and increased macromolecular permeability when adjusted to epithelia. However, other growth factors may have opposite results; (6) LTs[70] - Leukotriene D4 was found to induce reorganization of actin network and consequently affect TJs. There is evidence that phosphatidylinositol 3 kinase (PI3K) is involved in the signaling pathway used by LTD4; and (7) Glucocorticoids[71] - although still not completely understood, one study shows that they may destroy epithelial barrier by a mechanism of serine/threonine phosphorylation; oxidative stress: increased oxidative stress is associated with paracellular barrier dysfunction[72,73]; calcium level imbalance: low calcium is related to TJ disturbance[72,74].

Each stimulus affects TJs using a regulatory mechanism. So far the molecular basis of TJ regulation is partially known and continuous investigation reveals its complexity. Phosphorylation is probably the most common, but not the only regulatory pathway, and affects serine/threonine or tyrosine residues, usually with opposite results. Thus, serine/threonine high phosphorylation level is detected when TJ integrity and morphological stability is maximal, whereas low phosphorylation level on the same residues causes TJ dissociation and cytoplasmic localization of occludin. In contrast, tyrosine phosphorylation is related to TJ dissociation without cytoplasmic occludin localization[72,75-78], and block of tyrosine phosphorylation after TJ disruption leads to inability to reassemble[59]. An increasing number of protein kinases are detected to associate directly or indirectly with TJ intracellular components, including protein phosphatase 2A, PKC and atypical PCKs, CK2, PI3K, cAMP dependent kinase, Tyrosine kinases, Mitogen-activated protein kinases and protein tyrosine phosphatases. Another post-

Table 5 Interaction between bacterial strains and tight junctional molecules

Vibrio cholerae[87,88]	It expresses zonula occludens toxin that reversibly increases paracellular permeability, triggering phospholipase C and protein kinase Ca dependent actin polymerization This process is primary or secondary related to TJ disruption
Shigella flexneri[89]	Secretes heat stable proteins that affect intestinal cells and lead to TJ disruption, even in the absence of living bacteria
Clostridium perfringens[29,31,61,62]	Its enterotoxin interacts with high affinity to claudin-4, therefore also known as CPE-R Lower affinity receptors are claudin-3 and occludin. CPE is proposed to be a multifunctional toxin that first induces cell damage at the level of the cell membrane, and thereby relates to TJ proteins, causing structural and functional alterations[61] Michl *et al*[31] have studied the effect of CPE on pancreatic cell cancers that expressed claudin 4[31], and they suggest that targeting of claudin-4 expressing tumors with CPE can represent a promising treatment method
Clostridium difficile[90]	This pathogenic microorganism, known etiologic factor of pseudomembranous colitis, secretes two toxins TcdA and TcdB that act through the Rho GTPase pathway to produce cell damage Study for their effect on epithelial TJ structure assumed that they lead to actin rearrangement, actin-ZO1 dissociation and dissociation of TJ components with changes of their cytoplasmic localization[90]
EPEC[91-93]	EPEC secretes through the type Ⅲ secretion mechanism[87] the EspF protein, that is dose-dependently related to TER and epithelial barrier disruption and cytoplasmic localization of occludin[91] These effects seem to relate primary with phosphorylation of 20 kDa myosin light chain and cytoskeletal contraction. Occludin appears dephosphorylated on serine/threonine residues[92] The pathogenic action of EPEC on the intestinal epithelium is reversed by Saccharomyces boulardii[93]

CPE: Clostridium perfringens enterotoxin; CPE-R: Clostridium perfringens enterotoxin receptor; EPEC: Enteropathogenic *Escherichia coli*; TJ: Tight junction; ZO: Zonula occludens; GTPase: Guanosine triphosphatase.

translational mechanism that may participate in TJ regulation is N Glycozylation, occurring on CAR[79]. Proteolytic cleavage of occludin is mediated by PMNs during their transmigration and leads to a 22 kDa molecule without barrier-forming potential[63]. Finally, there is evidence that TJ composition can be regulated by transcription and translation modulation. For instance, symplekin and GATA-4 have been found to regulate TJ components such as claudin-2 and ZO-2[80-82].

TJs AT THE INTESTINAL BARRIER - CLINICAL IMPLICATIONS

General considerations and interactions

TJs are expressed by the intestinal columnar epithelium. As the TJs of every epithelial tissue, there are occludin and special claudins expressed at intestinal TJs that mainly regulate the permeability of the epithelial layer under normal conditions. TJ strands are copolymers of heterogeneous claudin species and occludin, and heterogeneous claudin species constitute the backbone of TJ strands *in situ*[24].

Occludin associated with ZO-1 in a linear strand is detected among the intestinal epithelia and is strictly related to the differentiation status[83]. In highly differentiated adenocarcinomas, occludin and ZO-1 are normally expressed and form TJ structures. On the contrary, low differentiation carcinomas are characterized by low or absent occludin expression, whereas ZO-1, although normally expressed, appears to concentrate at the inner membrane area in a dotted pattern.

The intestinal epithelium is in continuous contact with the microbial ecosystem of the gut, with which it establishes a dynamic relationship. Based mainly on culturing techniques it, had initially been estimated that more than 500 bacterial species inhabit the human gut[84]. With the advancement of metagenomic technology, the magnitude of bacterial species diversity was raised to 15 000 to 36 000 species based on rRNA sequence analysis[85]. A recent release of the data from the Metagenomics of the Human Intestinal Tract project revealed a total of 3.3 million non-redundant microbial genes in human fecal specimens[86]. Intestinal epithelial cells and bacteria interact in a continuous so-called "cross-talk", for mutual benefit. TJs participate in this interaction in a primary or a secondary way. They possess proteins that act as pathogen receptors and directly bind to the bacterial wall. Moreover, TJs can be secondarily affected by cytokine expression and by rearrangement of the actin cytoskeleton induced by other bacterial mechanisms, such as intimin-Tir mechanism of enteropathogenic *Escherichia coli*[87]. Some known interactions between bacterial strains and tight junctional macromolecules are described in (Table 5)[29,31,61,62,87-93]. Probiotics have a positive effect on TJ barrier, thus enhancing the epithelial resistance to pathogens, also reducing the paracellular permeability of antigens that may cause inflammation[94].

Dendritic cells, antigen presenting cells that belong to the subepithelial immune system, mediate a novel mechanism of bacterial uptake[95]. DCs send dendrites that express TJ proteins (occludin, claudin 1, ZO1) through the gaps between epithelial cells. These dendrites disassociate transcellular interactions, creating new ones between their surface proteins and previous cells. In this way, they send their dendrites to the epithelial surface and sample bacteria, without disrupting the integrity of the epithelial barrier.

Clinical implications

Several lines of evidence suggest that TJs are involved in the pathogenesis of inflammatory and non-inflammatory intestinal diseases. TJ disruption leads to an inadequate epithelial barrier and, subsequently, to an incontrollable water and electrolyte loss. Secondly, intestinal TJ disrup-

tion is implicated in the pathogenesis of diverse extraintestinal autoimmune and inflammatory diseases. Thirdly, intestinal TJs may be secondarily disrupted in the course of several intestinal or extraintestinal diseases, leading to their further aggravation through promotion of systemic responses to endotoxin escape from the gut lumen.

Pathogenetic role in intestinal diseases: Changes at the level of TJs are related to the inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis. These are diseases that display a course of recurrent exacerbation and subsidence periods. At the peak of the IBDs, polymorphonuclear neutrophils transmigrate through the epithelial layer and induce inflammation at the intestinal surface. Transmigration is accompanied by an increase of the epithelial permeability to ions and macromolecules, as a result of downregulation of TJ. More specifically, occludin seems to be downregulated in a mechanism different from that of other TJ proteins[96]. The observation that, in clinically asymptomatic Crohn's disease patients, increased intestinal epithelial permeability precedes clinical relapse by as much as 1 year, raised the assumption that permeability defect may be an early event in disease reactivation. Further evidence supporting that abnormal intestinal permeability occurs early in the pathogenesis of Crohn's disease is provided by a study demonstrating intestinal barrier disruption, even in the non-inflamed parts of ileum from patients with Crohn's disease[97]. The primary and potentially genetically determined association of intestinal TJs disruption with the evolution of IBD came from studies demonstrating increased gut permeability in otherwise healthy relatives of people with IBD[98]. However, the genetic factors implicated in these phenomena have not been revealed yet and the opposite opinion, that TJ disruption is a secondary event to the inflammatory process in IBD, is still under consideration[98,99]. Notably, disease exacerbations and the risk of developing IBD have been associated with emotional stress[100]. Experimental and clinical data provided evidence that psychological stress exerts injurious effects on intestinal TJs and increases gut permeability in IBD and irritable bowel syndrome[101,102]. Therefore, the pathogenetic role of intestinal TJs disruption in IBD beyond its potential genetic basis has an environmental stress-related component as well.

Celiac disease is an immune-mediated enteropathy triggered by an inappropriate T cell-mediated response to ingested gluten and its component gliadin. Clinical and experimental studies suggest that altered intestinal barrier function might play an inciting role in the development of celiac disease by allowing gliadin to cross the intestinal barrier and activate the immune system. Zonulin is the 47 kDa intestinal epithelial protein analogue of zonula occludens toxin (Zot) of Vibrio cholerae. Zonulin is normally expressed and secreted to the surface of intestinal and other epithelia (heart, brain). Zonulin and Zot have the same receptor on the cell surface and both trigger actin polymerization by PLC and PKCa pathways. Zonulin has been proposed to be the initial factor for the pathogenesis of celiac disease[87]. The pathogenetic role of intestinal TJs disruption in celiac disease is supported by studies demonstrating that increased intestinal permeability exists prior to disease onset, persists in asymptomatic patients who were on a gluten-free diet and is also present in a significant proportion of healthy first-degree relatives of patients with celiac disease who also have increased intestinal permeability[103-106].

Pathogenetic role in extraintestinal diseases: A combination of predisposing genetics, dysregulated intestinal barrier function and aberrant immune responses play an inciting role in type 1 diabetes. Increased gut permeability is believed to facilitate increased exposure to antigens that can trigger autoimmune destruction of the insulin-producing pancreatic beta cells[107]. Several lines of experimental and clinical data suggest that intestinal TJs disruption and increased gut permeability is an early event with a pathogenetic association with disease onset. Specifically, experimental studies with the Biobreeding diabetes-prone rat (BBDP), an inbred line in which autoimmune diabetes spontaneously develops when weaned onto a normal diet, showed that increased intestinal permeability, associated with decreased expression of the TJ protein claudin-1, precede the onset of insulitis and clinical diabetes[108]. Also, increased intestinal permeability was found in diabetic patients at various stages of disease progression and their relatives; however, prediabetic subjects had the greatest increase[109,110]. Zonulin has been proposed to be the initial factor for the pathogenesis of diabetes mellitus type 1[110].

Food allergies are expressed as adverse multisystemic immune-mediated reactions to ingested food proteins/antigens. Current pathogenetic aspects highlight the pivotal role of increased intestinal permeability, which permits increased dietary antigen transport across the intestinal barrier and exposure of dietary antigens to the mucosal immune system, leading to the development of the dietary antigen-specific response. In support of this hypothesis, intestinal permeability in infants with food allergies was significantly increased compared with that seen in healthy children[111]. In subjects with food allergies, intestinal permeability remained increased even after 6 mo of an allergen-free diet, indicating that the increased permeability probably preexists and is independent of food antigen stimulation[112]. Additional data supporting a role for increased intestinal permeability in the development of food antigen sensitization and food allergies are provided by recent clinical studies that demonstrate an association between increased intestinal permeability and the development of new-onset food allergies in patients after liver and heart transplantation under immunosuppressant therapy. These allergies were not related to the passive transfer of food antigen-specific IgE or lymphocytes from donors to previously nonallergic recipients, as were developed even in cases of non-allergic donors[113,114]. It is considered that immunosuppressive agents disrupt intestinal TJs, thus increasing permeability

and facilitating presentation of food allergens to the immune system[115].

Beyond diabetes type 1, a similar mechanism has been proposed to contribute to the pathogenesis of diverse autoimmune diseases like atopic dermatitis, ankylosing spondylitis, Hashimoto's thyroiditis and autoimmune hepatitis. The classical paradigm of autoimmune pathogenesis involving specific gene makeup and exposure to environmental triggers has been recently challenged by the addition of a third element, the loss of intestinal barrier function[116]. Disruption of intestinal TJs in genetically predisposed subjects might trigger a pathological immune response. According to this theory, once the autoimmune process is activated, it is not auto-perpetuating, but rather can be modulated or even reversed by preventing the continuous interplay between genes and environment, or in other words, by preventing loss of gut barrier function[116]. A zonulin - induced intestinal TJs disruption has been proposed as the responsible mechanism of barrier dysfunction allowing antigens to invade subepithelial and cause autoimmune reactions[87].

Secondarily affected in intestinal and extraintestinal diseases: Diverse intestinal and extraintestinal diseases during their course exert injurious effects on the integrity of intestinal TJs. Disruption of intestinal barrier function further subsequently aggravates through promotion of a systemic inflammatory response and the structural and functional integrity of the diseased and other organs, leading to clinical deterioration. Thus, although secondarily affected, dysfunction of the gut barrier exerts a pivotal role for the clinical outcome of diverse diseases. There are two main theories of how this may occur. The first one supports the view that after an initial insult, the intestinal barrier is compromised, thus allowing the passage of intestinal bacteria and endotoxins in mesenteric lymph nodes, portal circulation and normally sterile extraintestinal tissues (bacterial translocation)[117]. This process causes systemic infections and promotes a systemic inflammatory response both associated with distant organ failure and development of a septic state[118]. The second theory supports that, after the initial injurious insult and disruption of gut barrier integrity, bacteria and endotoxins crossing the mucosal barrier activate an intestinal inflammatory response, even when these translocating factors are trapped within the gut wall or intestinal lymph nodes and do not reach the systemic circulation[119]. Thus, the gut becomes a proinflammatory organ and gut-derived inflammatory factors, carried mainly in mesenteric lymph, induce a systemic inflammatory response and multiple organ failure[119-121]. In both theories, the intestinal TJs are further disrupted under the influence of systemic cytokinemia, further aggravating intestinal barrier function and leading to a vicious cycle.

The above described mechanisms may occur in diverse clinical states such as in intestinal ischemia[122], hemorrhagic shock[123,124], in critically ill patients[125], total parenteral nutrition[126], radiation enteritis[127], burns[128], ileus[129], acute pancreatitis[130], sepsis[131], cardiac bypass surgery[132], chemotherapy[133], obstructive jaundice[134,135], alcoholic liver disease[136], liver resections[137,138] and liver cirrhosis[139-141].

FUTURE DIRECTIONS

Protection against external stimuli and challenging factors is mainly offered by the skin with its several layers, containing cells at different levels of differentiation, in order to better serve this function. In an analogous way, the epithelium covering the gastro-intestinal tract protects against several "foreign factors" whether they may be chemicals, microorganisms or of a different nature. At the same time, the intestine has to serve an equally important opposite function; the selective permeability of needed nutrients from the intestinal lumen into the circulation and into the internal milieu in general. These essential life-sustaining opposite functions are performed in the intestine almost exclusively by a monolayer epithelium, the intestinal epithelium, and one of the key elements, if not the most important for this "compartmentalization" function, is the presence and the versatility of existing TJs.

The study of the structure of TJs has led to exciting new information and now over 40 macromolecular components are in the picture. However, we still lack detailed information about the mode of extracellular associations between occludin and claudins and the mechanism(s) by which they form a tight seal between cell membranes. The fact that several molecular combinations exist in different tissues certainly suggests that there is extremely fine tuning in determination of paracellular permeability, in a highly tissue-specific manner. Information is also missing regarding the details of assembly and disassembly of TJs and the exact role of each factor affecting the phenomena. Further understanding of these processes will allow us to design pharmacological interventions with impressive tissue specificity and will endow us with a better understanding of several pathogenetic mechanisms at the molecular level. Pharmacological modulation of intestinal permeability in a specific manner will enable us to treat and even prevent several intestinal and extraintestinal diseases, possibly overcoming the issue of severe side effects observed with current treatment modalities. Furthermore, understanding the specific differences in nucleotide/protein sequences of tight junctional macromolecules among individuals will allow the establishment of "personalized medicine" therapeutic modalities in the future.

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ORIGINAL ARTICLE

Effect of fiber supplementation on the microbiota in critically ill patients

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Abstract

AIM: To determine tolerance to fiber supplementation of semi-elemental tube feeds in critically ill patients and measure its effect on colonic microbiota and fermentation.

METHODS: Thirteen intensive care unit patients receiving jejunal feeding with a semi-elemental diet for predominantly necrotizing pancreatitis were studied. The study was divided into 2 parts: first, short-term (3-9 d) clinical tolerance and colonic fermentation as assessed by fecal short chain fatty acid (SCFA) concentrations and breath hydrogen and methane was measured in response to progressive fiber supplementation increasing from 4 g tid up to normal requirement levels of 8 g tid; second, 4 patients with diarrhea were studied for 2-5 wk with maximal supplementation to additionally assess its influence on fecal microbiota quantitated by quantitative polymerase chain reaction (qPCR) of microbial 16S rRNA genes and Human Intestinal Tract Chip (HITChip) microarray analysis. Nearly all patients were receiving antibiotics (10/13) and acid suppressants (11/13) at some stage during the studies.

RESULTS: In group 1, tolerance to progressive fiber supplementation was good with breath hydrogen and methane evidence (P = 0.008 and P < 0.0001, respectively) of increased fermentation with no exacerbation of abdominal symptoms and resolution of diarrhea in 2 of 4 patients. In group 2 before supplementation, fecal microbiota mass and their metabolites, SCFA, were dramatically lower in patients compared to healthy volunteers. From qPCR and HITChip analyses we calculated that there was a 97% reduction in the predominant potential butyrate producers and starch degraders. Following 2-5 wk of fiber supplementation there was a significant increase in fecal SCFA (acetate 28.4 ± 4.1 μmol/g to 42.5 ± 3.1 μmol/g dry weight, P = 0.01; propionate 1.6 ± 0.5 *vs* 6.22 ± 1.1, P = 0.006 and butyrate 2.5 ± 0.6 *vs* 5.9 ± 1.1, P = 0.04) and microbial counts of specific butyrate producers, with resolution of diarrhea in 3 of 4 patients.

CONCLUSION: Conventional management of critically ill patients, which includes the use of elemental diets and broad-spectrum antibiotics, was associated with gross suppression of the colonic microbiota and their production of essential colonic fuels, i.e., SCFA. Our investigations show that fiber supplementation of the feeds has the potential to improve microbiota mass and function, thereby reducing the risks of diarrhea due to dysbiosis.

Key words: Critical illness; Acute pancreatitis; Microbiota; Enteral nutrition; Fiber

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INTRODUCTION

Diarrhea is a frequent complication in critically ill patients and often results in interruption of feeding and exacerbation of malnutrition when it is associated with tube feeding. For example, we recently reported our experience with acute pancreatitis where 43% of tube-fed patients developed persistent diarrhea[1,2]. Whilst diarrhea in tube-fed patients was previously attributed to feed-intolerance and malabsorption by the small intestine, it has become increasingly recognized that the major cause is antibiotic disturbance of the microbiota, termed "antibiotic-associated diarrhea" or "dysbiosis"[3-5]. This disturbance may precipitate diarrhea by at least two mechanisms: by suppressing fermentation and reducing the production of the primary energy source and epithelial regulator of the colonic mucosa - the short chain fatty acid (SCFA), butyrate, and by permitting the overgrowth of pathogens. Perhaps the best recognized example of the second mechanism is *Clostridium difficile* (*C. difficile*) infection. In our clinical study mentioned above, 50% of the cases of diarrhea were associated with *C. difficile* infections[2]. The emergence of *C. difficile* as a notorious "superbug" responsible for epidemics of hospital-acquired infections worldwide has attracted major media concern (e.g., "Stomach Bug Crystallizes an Antibiotic Threat", New York Times, April 13, 2009). There is good evidence that *C. difficile* infection is a consequence of dysbiosis as it thrives in a "permissive" environment[4,5] devoid of butyrate[6], indeed its presence may be a biomarker of the severity of the dysbiosis.

We recently hypothesized that current intensive care unit (ICU) management which invariably includes broad-spectrum antibiotic therapy, proton pump inhibitors (PPI) and elemental tube feeds, forms an ideal environment for the proliferation of *C. difficile* infection[7]. We are concerned that research into enteral nutrition has focused on the needs of the upper gastrointestinal (GI) tract with the development of specialized feeds that enhance enterocyte function but starve the colon as they are fully absorbed in the small intestine (i.e. non-residual, elemental). "Topical" nutrition is essential for health in not only the small intestine, but also the large, where undigested complex carbohydrates support microbiota health and balance, which in turn produce SCFAs and butyrate, which maintain mucosal function and health. As there is good evidence that fiber supplementation of tube feeds can reduce diarrhea in critically ill septic patients in the ICU receiving antibiotics[8] and in patients receiving enteral nutrition for severe acute pancreatitis[9,10], we conducted the following study to (1) test tolerance to progressive fiber supplementation; and (2) examine the effect this had on the microbiota and their production of SCFAs in critically ill patients, predominantly with acute pancreatitis, needing enteral feeding.

MATERIALS AND METHODS

Study design

The study was divided into 2 parts. In the first group of critically ill patients (group 1), short-term (3-9 d) clinical tolerance and colonic fermentation responses to progressive fiber supplementation of their elemental tube feeds was measured. In the second part, a smaller number of "high-risk" severely ill ventilated patients, all dependent on jejunal feeding and all with diarrhea (group 2), were followed for a longer period of time (2-5 wk) to assess not only tolerance but also the associated changes in microbiota composition. Results were evaluated by comparison to age and sex-matched healthy volunteers consuming a normal diet.

Patient selection: Adult critically ill ICU patients referred to our nutrition support service with impaired gastric emptying for jejunal feeding with semi-elemental diets were selected. Patients were only included if it was estimated that they would need long term feeding, i.e. more than 2 wk. As reflected by our practice, most of the patients needed jejunal feeding for acute pancreatitis complicated by cystic swelling or necrosis, causing gastro-duodenal compression. Details of our nutritional management of this group of patients with a double-lumen gastric decompression and jejunal feeding tube has recently been published[11-13].

Controls: To evaluate the fermentation responses to fiber and fecal microbiota composition, 5 age-matched healthy subjects, body mass index range 23-27 kg/m^2, served as controls. Breath hydrogen responses to drinks containing 10 g of soluble fiber were measured hourly for 6 h following the drink. Fresh morning stool samples were obtained from the same individuals consuming normal food for characterization of the microbiota composition and activity. For fecal SCFA concentrations, we used our recently published data from twenty-three 50-65-year-old healthy male and female Americans also consuming a normal diet[14].

Enteral feeding: Jejunal feeding was commenced and managed as previously described[11]. Transnasal endosco-

py (Olympus 5.8 mm diameter upper GI endoscope) was used to access the jejunum to place a guide wire down the jejunum, permitting tube placement after withdrawal of the endoscope[12]. A double-lumen nasogastric decompression-jejunal feeding tube was used in all cases (Kangaroo-Dobbhoff tube system, Sherwood Medical Co., St Louis, MI; 16 Fr outer gastric tube, 9 Fr internal jejunal feeding tube). Feeding was commenced using a semi-elemental formula diet (Peptamen AF, Nestle Nutrition, NJ, United States, which contains 4 g soluble fiber (as oligofructose and inulin) per liter at 25 mL/h for 24 h, then increased to 50 mL/h for day 2, and then to goal calculated as the volume needed to deliver 1.5 g protein/kg ideal body weight/day. It is important to note that the recommended intake of fiber for Americans is 25 g/d for women and 38 g/d for men[15].

Fiber supplementation: In both groups, fiber supplementation was only commenced after goal enteral nutrition infusion rates had been achieved. In group 1, baseline breath responses to conventional tube feeding were measured at hourly intervals during the day (roughly 9 am to 5 pm) before fiber supplementation. On the following day, the response to bolus injection of 10 g of the fiber supplement (Benefiber, Novartis, United States, a wheat dextrin, dissolved in 25 mL water) down the jejunal tube was measured at hourly intervals for 6 h. Progressive fiber supplementation of the tube feeds was then commenced at 4 g tid, increasing after successive 3-d periods with tolerance to 8 g tid and up to a maximum of 12 g tid if diarrhea continued. Tolerance was assessed clinically with particular attention to abdominal distress, namely nausea, vomiting, diarrhea, abdominal distention, gas and pain. Ventilated patients were assessed by abdominal examination for pain and gaseous distention and diarrhea. Diarrhea was defined by our own practice protocol as the passage of more than 3 liquid stools > 500 mL/d; severe diarrhea was defined as the passage of > 1000 mL/d. Fermentation was monitored by daily measurement of breath hydrogen and methane at hourly intervals between 9 am and 5 pm. Fermentation was also assessed by measurement of fecal SCFA concentrations before and on the last day of fiber supplementation. In group 2, progressive fiber supplementation was given in a similar way but for a longer duration (2-5 wk) and as a more physiological continuous infusion piggy-backed into the jejunal feeding tube.

Sample collection

Most fecal samples, and in particular those collected from ventilated patients, were obtained from rectal tubes. Samples were contained in 10 g airtight sterile containers and frozen at -80℃ immediately after defecation. End expiratory breath was sampled and analyzed as previously[16].

Microbiota analysis

The microbiota was assessed in all patients in group 2 before and after fiber supplementation by estimating counts of total and specific bacteria by real time quantitative polymerase chain reaction (qPCR) of the 16S ribosomal RNA gene using Bacteria domain primers (broad primers Uni331F and Uin797R)[17] and Bifidobacteria (primers Bif164F, Bif662R) (Sigma Aldrich, CA). All PCR experiments were done in triplicate with a reaction volume of 10 μL using MicroAmp optical 384-well reaction plates. Following amplification, a dissociation step was included to analyze the melting profile of the amplified products. Ten-fold dilution series of the plasmid standard for the respective bacterial group or species was run along with the samples. Quantification of unknowns was made by using standard curves obtained from the amplification profile of known concentrations of the plasmid DNA containing the respective amplicon for each set of primers. Data were analyzed with SDS v2.3 software supplied by Applied Biosystems.

In 2 patients and 2 of the controls (1 male, 1 female), full analysis of microbial composition was determined by phylogenetic microarray using the Human Intestinal Tract Chip (HITChip) at Wageningen University in The Netherlands. This provides information on the proportional composition on over 1100 intestinal bacterial phylotypes[18]. Proportions were converted to counts by measuring counts of total bacteria in fecal samples as above. Confirmation of these calculations was made by also measuring counts of specific bacterial taxa by qPCR. SCFA were measured in duplicate samples as previously described[14] by gas chromatography (Agilent Technologies 6890N Network GC System with flame-ionization detection).

Statistical analysis

Baseline values from patients were evaluated employing computer software (SPSS 17 for Windows) by comparison to healthy controls by Student's unpaired *t* test if normally distributed and by Mann-Whitney *U* test if not. Changes after supplementation were evaluated by Students paired *t* test or Wilcoxon Rank Sum test as appropriate.

RESULTS

Patients

Thirteen patients were enrolled, 9 in group 1 and 4 in group 2. Demographic details are summarized in Table 1. The majority needed jejunal feeding for the management of complicated severe necrotizing pancreatitis and organ failure (all with Apache II scores > 20) where gastric feeding was impossible because of gastro-duodenal compression by the inflammatory mass, and distal elemental diet feeding was appropriate to maintain gut function without pancreatic stimulation[19,20]. One patient had multiple traumas due to a motor vehicle accident and another was started on tube feeding for *C. difficile* colitis and gastroparesis. All but one were commenced on fiber supplementation within the first week of commencement of feeding. All but 3 patients were receiving prophylac-

Table 1 Demographic details of the patients studied, with outline of medications received and outcome

Patient	Diagnosis	Age (yr)	Sex	BMI (kg/m^2)	Duration of fiber suppl (d)	Maximum fiber (g/d)	Medications, gastrointestinal symptoms before feeding, outcome
Group 1: bolus injections: short term							
1	Trauma	59	M	34.7	3	15	Metronidazole, lanzoprazole, diarrhea, d/c to snf
2	SAP	65	M	38.0	8	29	Cefepime, lanzoprazole, diarrhea, discharge home
3	SAP	85	F	31.0	3	24	Omeprazole, diarrhea, discharge home
4	SAP	89	F	29.4	6	32	Fluconazole, lanzoprazole, transfer rehab
5	SAP	43	M	34.7	9	12	Fluconazole, vancomycin, pantoprazole, discharge home
6	SAP	34	M	44.8	3	18	Ertapenem, omeprazole, diarrhea, d/c to snf
7	SAP	81	F	35.5	3	15	Omeprazole, discharge home
8	SAP	62	M	27.0	7	22	Famotidine, metronidazolel, discharge home
9	SAP	56	M	28.7	6	24	Trimethoprim-sulfamethoxazole, voriconazole, pantoprazole, discharge home
Group 2: continuous infusions: long term							
10	SAP	88	F	30.3	19	35	Metronidazole, aztreonam, famotidine, diarrhea transfer to snf
11	SAP	65	F	27.6	36	36	Piperacillin-tazobactam, doripenem, metronidazole, pantoprazole, cefuroxime, diarrhea, distension, pain. d/c to snf
12	SAP	47	F	31.0	33	18	Piperacillin-tazobactam, pantoprazole, fluconazole, metronidazole, vancomycin, diarrhea, distention, d/c to snf
13	Chronic sepsis C diff	79	F	34.9	23	24	Metronidazole, vancomycin, lanzoprazole, diarrhea, distension, pain. *Clostridium difficile*. d/c to snf

SAP: Severe acute pancreatitis; d/c: Discharge; snf: Skilled nursing facility; BMI: Body mass index.

tic broad-spectrum antibiotics during the fermentation measurements. All but 2 were also receiving acid suppressants, 9 on PPI and 2 on H-2 antagonists. Six made an otherwise uneventful recovery, were weaned back onto a normal diet and discharged home, but 7 continued to need tube feeding due to continued gastric outlet compression, and were transferred once in a stable condition to a rehabilitation unit or specialized nursing facility.

Group 1

Tolerance: Tolerance to bolus fiber supplementation overall was good, with a median supplementation rate of 22 g/d, range 12-32 g/d. For the 5 who had no diarrhea before commencement of supplementation, supplementation rates of 15-32 g/d were tolerated without the induction of GI symptoms. In the 4 patients who had diarrhea at commencement of fiber supplementation, 2 improved with supplementation of 18 and 24 g/d and 2 remained unchanged with supplementation of 15 and 29 g/d. Gas was not a complaint and increasing abdominal distension was not detected clinically.

Fermentation: Figure 1 shows a comparison between the normal breath hydrogen responses to consumption of 10 g soluble fiber in the 5 healthy volunteers and the 9 patients in group 1 at the commencement of bolus supplementation. The normal increase in breath hydrogen over 6 h was not seen, chiefly because initial hydrogen concentrations were very high in 2 subjects (186 and 113 ppm), suggesting premature fermentation of tube feeds and soluble fiber by bacterial overgrowth of the small intestine[21].

Figure 2 summarizes the fecal SCFA results of the responses to maximal fiber supplementation in group 1. Although the group mean fecal SCFA concentrations

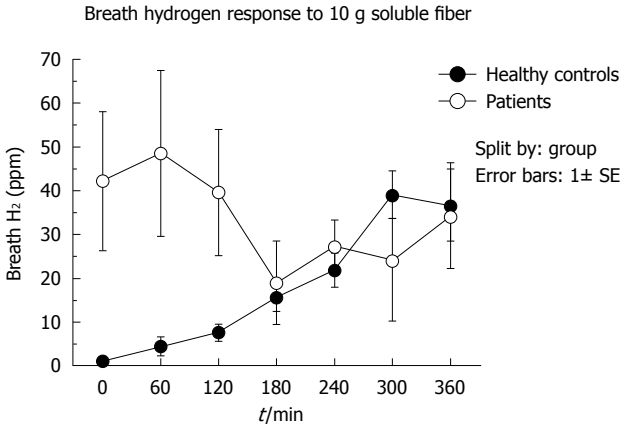

Figure 1 Comparison of breath hydrogen responses over 360 min to a bolus of 10 g soluble fiber between group 1 and healthy subjects.

increased after supplementation, the individual changes were variable and therefore not statistically significant. Both breath hydrogen and breath methane increased significantly ($P = 0.008$ and $P < 0.0001$ respectively).

Group 2

Effects of 2-5 wk fiber supplementation: All 4 of these patients had diarrhea at commencement of supplementation. In 3, the diarrhea improved with progressive supplementation to 18, 24, and 35 g/d. In the fourth patient (No. 11), with severe acute necrotizing pancreatitis, the diarrhea continued despite progressive supplementation to 36 g/d. This patient differed from the others as she continued to need broad-spectrum IV antibiotics (cefuroxime) and PPI (pantoprazole) for suspected sepsis and high gastric juice drainage in contrast to 2 of the other patients (No. 10 and No. 13) who were weaned off

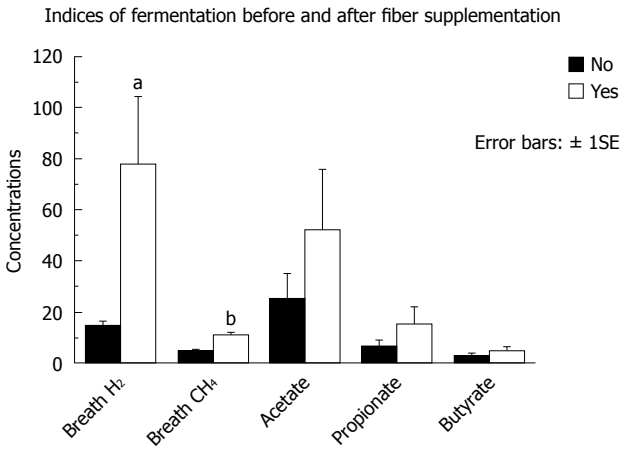

Figure 2 Summary of the changes in some of the indices of bacterial fermentation observed in the first group of 9 tube fed critically ill patients (group 1) after achievement of maximal fiber supplementation (median 22 g/d, range 12-32 g/d). $^{a}P = 0.008$, $^{b}P < 0.0001$, unpaired Student's *t* test.

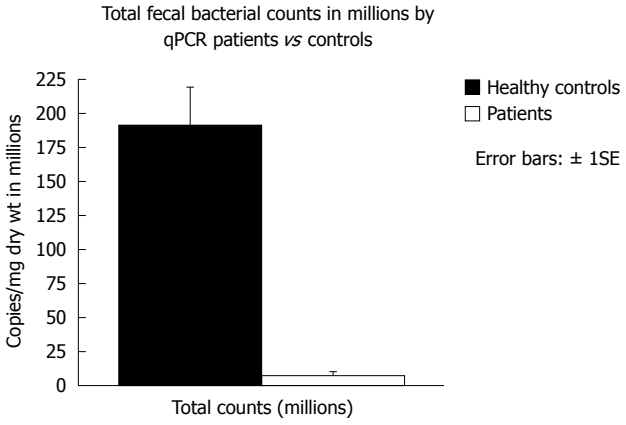

Figure 3 Comparison of total fecal bacterial copies in group 2 compared to healthy subjects consuming normal food measured by quantitative polymerase chain reaction of the 16S ribosomal RNA gene using Bacteria domain primers. qPCR: Quantitative polymerase chain reaction.

antibiotics and PPI before the end of the study. The third patient (No. 12), whose diarrhea settled on 18 g/d fiber supplementation, was still on IV vancomycin after diarrhea resolution.

Fecal microbiota and SCFAs: Figure 3 shows that total fecal bacterial counts estimated by qPCR were orders of magnitude lower in the 4 patients compared to healthy controls. Commensurate with this, fecal SCFA were also significantly lower in patients (Figure 4). Composition analysis by HITChip in 2 of the 4 patients (No. 10 and No. 11) and 2 healthy controls showed that the composition of the remaining microbiota was also different (Figure 5). The difference in Bacteroidetes composition was striking, with this phylum making up 35% of the microbiota in healthy subjects and 60% in patients. Conversely, there was a reduction of the proportion of Firmicutes, which contain the major butyrate-producers, in patients (50%) compared to controls (30%). Converting the proportions of phyla to numbers by combining the qPCR and HITChip

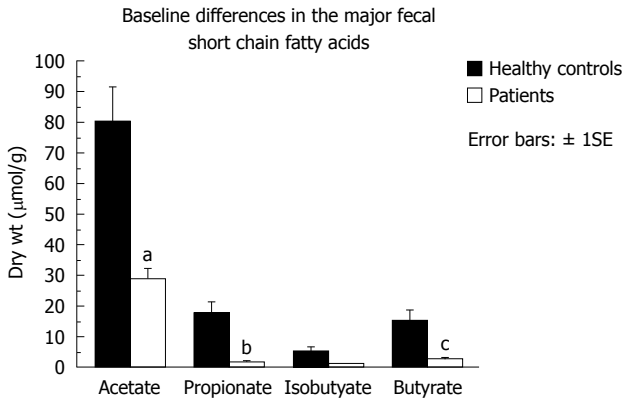

Figure 4 Fecal short chain fatty acid concentrations were significantly lower in the 4 patients in group 2 given fiber supplementation for longer periods of time (2-5 wk) compared to healthy subjects. $^{a}P = 0.012$, $^{b}P = 0.007$, $^{c}P = 0.35$, unpaired Student's *t* test.

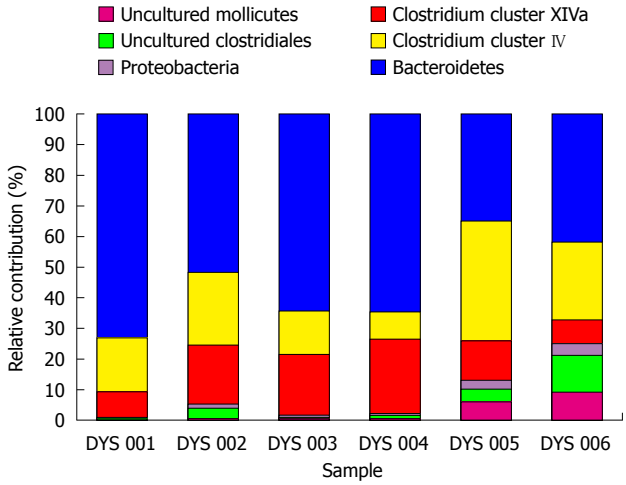

Figure 5 Phylogenetic distribution at level 1 ("Phylum/Class" level) by HITChip analysis. Lentispaerea is the spike. This illustrates differences in the composition of the major phyla between 2 patients from Group 2 before and after fiber supplementation (DYS 001 and DYS 002 = patient No. 10 before and after fiber supplementation, DYS 003 and DYS 004 = patient No. 11 before and after fiber supplementation) and 2 of the healthy controls consuming normal food (DYS 005 and DYS 006). The difference in *Bacteroidetes* composition was striking, with this phylum making up 35% of the microbiota in healthy subjects and 60% in patients. Conversely, there was a reduction of the proportion of *Firmicutes* (*Clostridium* clusters IV and XIVa), which contain the major butyrate-producers, in patients (50%) compared to controls (30%).

analyses, we calculate that there was a 97% reduction in the predominant potential butyrate producers and starch degraders, at the genus level, from *Clostridia* clusters XIVa [*Eubacterium rectale* (*E. rectale*), *Roseburia intestinalis* (*R. intestinalis*)] and IV [*Faecalibacterium prausnitzii* (*F. prausnitzii*), *Ruminococcus bromii* (*R. bromii*) and *Ruminococcus obeum* (*R. obeum*)] before fiber supplementation. Taken together with the lower fecal SCFA concentrations, this demonstrates a general suppression of colonic fermentation.

Following 2-5 wk fiber supplementation in group 2, there was a 6-fold increase in Firmicutes accompanied by a significant increase in fecal SCFA (acetate 28.4 ± 4.1 μmol/g to 42.5 ±3.1 μmol/g dry weight, $P = 0.01$; pro-

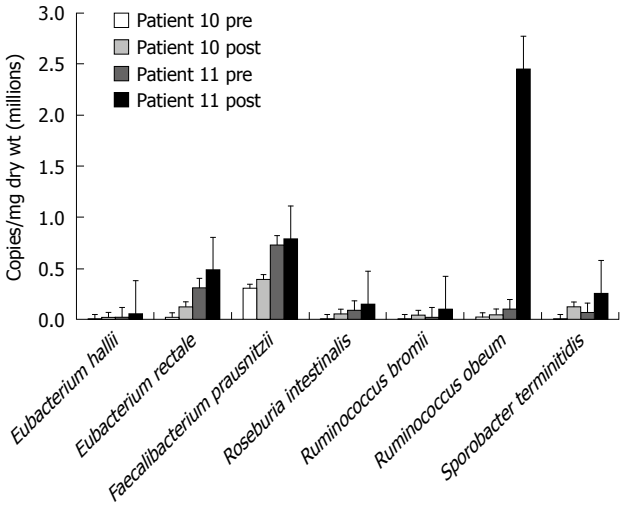

Figure 6 Numbers of butyrate-producing and fiber-digesting bacteria based on HITChip phylogenetic microarray analysis of microbiota composition and *16S* quantitative polymerase chain reaction of total fecal bacterial counts in two critically ill patients showing a general increase before and after 2-5 wk of fiber supplementation.

pionate 1.6 ± 0.5 *vs* 6.22 ± 1.1, $P = 0.006$; and butyrate 2.5 ± 0.6 *vs* 5.9±1.1, $P = 0.04$), indicating an increase in carbohydrate fermentation. The responses of a select number of bacteria known to maintain colonic mucosal health are illustrated in Figure 6. In general, all of these bacteria increased in numbers after supplementation, more so in patient 11 than in patient 10 - which might be explained by the fact that patient 10 was still receiving antibiotics during the repeat measurement during fiber supplementation. Specifically, microbial counts increased with fiber supplementation in the major potential butyrate producers, *E. rectale, E. hallii* and *R. intestinalis*, all members of Clostridia cluster XIVa. There were similar increases in *R. bromii, R. obeum* and in *Sporobacter terminitidis*, organisms that degrade starch and other complex carbohydrates. *F. prausnitzii* counts also increased in both patients. This bacterium is not only involved in starch degradation but may also have a specific role in preventing colitis[22]. The "probiotic" genus, Bifidobacterium spp. also increased towards normal (1.5×10^5 to 5.9×10^5, control 8.4×10^5 by qPCR).

DISCUSSION

The present data demonstrate that, in the patient population studied, progressive supplementation of elemental formula diets with up to 35 g soluble fiber per day fed into the jejunum was well tolerated, with no increase in abdominal symptoms and resolution of diarrhea in 5 of 8 patients. Secondly, detailed investigation in a few of these subjects fed for 3-5 wk revealed that initial fecal bacterial counts were dramatically reduced compared to healthy volunteers and that fiber supplementation increased the proportions and counts of recognized butyrate-producers as well as fecal butyrate concentrations. However, it was important to note that levels remained lower than normal, possibly because of the effects of concomitant antibiotic therapy. We acknowledge that our microbiota findings must be considered provisional, as the few patients we studied - because of cost of analysis - may not be representative of all ICU patients. Nonetheless, it is very likely that the widespread use of antibiotics and fiber-deficient diets have a devastating effect on microbiota composition and function placing patients at high risk of diarrheal disease.

Most of the patients studied were dependent on jejunal tube feeding because of complications of severe acute pancreatitis as this population reflects the bulk of our nutritional support practice commonly needing prolonged ICU support and tube feeding[1,2]. The results demonstrate that nearly all were managed with broad-spectrum antibiotics and acid suppressants, all received low residue semi-elemental tube feeds and most (8/11) had diarrhea when first studied. In a systematic review of the literature, Petrov and Whelan identified only 5 suitable randomized controlled trials (RCT) comparing the frequency of diarrhea in enteral and parenterally fed patients with acute pancreatitis[9]. Diarrhea was significantly higher in enterally fed patients: 29% *vs* 7%; but this difference is to be expected if the gut is not being used. There is only one RCT evaluating the value of fiber supplementation of the enteral feeds, that of Karakan *et al*[10] who randomized 30 patients with severe acute pancreatitis (mean Apache Ⅱ score of 9.4-9.6) to supplementation with 24 g/d of soluble (0.7 g/100 mL) and insoluble fiber (0.8 g/100 mL). Remarkably, supplementation was associated with a significant reduction in hospital stay, duration of nutrition therapy, acute phase response and overall complications.

The maintenance of microbiotal balance is pivotal for colonic health as they provide essential nutrients for the colonic mucosa[23]. Ever since Roediger[24] first identified butyrate as the preferred energy substrate for colonocytes and Harig *et al*[25] showed that depletion of colonic SCFA lead to the development of acute colitis ("diversion colitis"), there has been intense, but chiefly experimental, research into the importance of SCFA synthesis in the maintenance of colonic health. We have recently reviewed some of the more recent evidence, which indicates that sufficient colonic butyrate is essential for the maintenance of cellular homeostasis and a normal colonocyte phenotype; that SCFA have anti-inflammatory effects, either directly by regulating the release of prostaglandin E2, cytokines and chemokines from human immune cells, or indirectly by their ability to support the growth of probiotic species, such as Bifidobacteria and Lactobacilli; that SCFA also possess immune-modulating and anti-inflammatory actions by binding certain G-coupled receptors, which may stimulate the normal resolution of inflammatory responses in colonocytes; and finally, that SCFA production enhances colonic blood flow as well as fluid and electrolyte uptake[26].

However, butyrate production is not the only essential function of the microbiota. Water-soluble vitamins, such as folate, biotin, B-12, are also synthesized and utilized by

the mucosa[22] and different microbial species support each other with cross-feeding, thus maintaining a disciplined society preventing overgrowth with pathogens such as *C. difficile*. For example, starch degraders such as *R. bromii* and *Bifidobacterium adolescentis* cross-feed to produce acetate, which is the major energy source for the major butyrate-producers *E. rectale*, *Roseburia* spp. and *F. prausnitzii*[27]. Bifidobacteria also process starch and soluble fiber into lactate, which is released into the lumen and fuels other butyrate-producers such as *E. hallii*[27]. Furthermore, the lactate reduces the luminal pH, favoring the growth of butyrate-producers and suppressing pathogens. The real-life situation is likely far more complex than we currently recognize, bearing in mind that the microbial population outnumbers our own cells by 10:1 and that their genetic library outnumbers ours by 100:1[28]. For example, specific bacteria may secrete specific anti-inflammatory substances as illustrated by Sokol *et al*[22], with the observation that *F. prausnitzii* exhibits anti-inflammatory effects on cellular and TNBS colitis models, partly due to secreted metabolites able to block nuclear factor κB activation and interleukin-8 production.

Although we did not directly measure microbial activity in the small intestine, we performed soluble fiber-breath tests, which provide indirect information on the distribution of fermenters in the small and large intestine. The high baseline and early increase in breath hydrogen levels after injection of soluble fiber into the jejunum shown in Figure 1, is characteristic of small bowel bacterial overgrowth, as the tube feed and fiber is fermented before it can be absorbed in the small intestine or fermented in the colon. The most likely explanation for bacterial overgrowth of the small intestine is the virtual routine use of PPI in critically ill patients. Earlier studies of ours, in 20 patients with peptic ulcer disease before and after PPI therapy (omeprazole 20 mg/d), showed that duodenal bacterial counts increased in all patients following treatment, geometric mean counts increasing from 330 CFU/mL to 95 000 CFU/mL, and that this was accompanied by an increase in intestinal transit and diarrhea[21].

Our investigations raise serious concern that the normal microbiota balance within us is grossly disturbed in critically ill patients managed with semi-elemental tube feeds, acid suppressants and antibiotics. Not only are the microbiota killed or suppressed by broad-spectrum antibiotics, but the remaining colonies are starved by the use of non-residue diets. This combination inevitably leads to dysbiosis and increased risk of colitis and diarrhea. Further studies are needed to separate out the relative risks due to antibiotics and colonic starvation and to determine whether the incremental improvement in microbial growth and function with fiber supplementation will be sufficient to counteract the effects of antibiotic therapy. At the same time, the use of unproven antibiotic (as in patients with acute pancreatitis[29]) and PPI prophylaxis[30-32] should be withheld, or modified, so that it spares colonic health-promoting bacteria, as in the case of the new anti-*C. difficile* drug, fidaxomicin[33].

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COMMENTS

Background

Diarrhea is a common problem in critically ill patients dependent on tube feeding for prolonged periods. There is concern that the diarrhea results from the combined use of elemental formulae diets which deprive the colonic microbiota of their nutrition, which in turn reduce the production of short chain fatty acids (SCFAs) which maintain the health of the colonic mucosa, and the regular use of broad spectrum antibiotics which further suppress microbial fermentation.

Research frontiers

Preservation of colonic microbiota composition and function is likely to reduce the morbidity associated with conventional intensive care unit (ICU) management.

Innovations and breakthroughs

The studies have demonstrated that progressive supplementation of jejunal tube-feeds with soluble fiber is well tolerated by critically ill patients, increasing colonic fermentation and reducing diarrhea. High throughput technology was employed to investigate the nature of the underlying dysbiosis. First, a phylogenetic microarray using the Human Intestinal Tract Chip provided information on the proportional composition on over 1100 intestinal bacterial phylotypes. Second, proportions within the microbiota composition were converted to counts by measurement of the total bacterial counts in fecal samples by quantitative polymerase chain reaction analysis.

Applications

The results indicate that patients dependent on semi-elemental diets for any length of time should be given regular fiber supplementation to maintain colonic health and function.

Terminology

Fiber is a complex carbohydrate that is indigestible by small intestinal enzymes but fermentable by colonic microbes to form SCFAs, which are the primary energy source and epithelial regulators for the colonic mucosa.

Peer review

This is a unique study examining the interactions between the conventional ICU management, including semi-elemental diets and antibiotics, and the composition and function of the colonic microbiota.

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ORIGINAL ARTICLE

Stimulation of oval cell and hepatocyte proliferation by exogenous bombesin and neurotensin in partially hepatectomized rats

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Abstract

AIM: To investigate the effect of the neuropeptides bombesin (BBS) and neurotensin (NT) on oval cell proliferation in partially hepatectomized rats not pretreated with a known hepatocyte inhibitor.

METHODS: Seventy male Wistar rats were randomly divided into five groups: Ⅰ = controls, Ⅱ = sham operated, Ⅲ = partial hepatectomy 70% (PHx), Ⅳ = PHx + BBS (30 μg/kg per day), Ⅴ = PHx + NT (300 μg/kg per day). Forty eight hours after liver resection, portal endotoxin levels and hepatic glutathione redox state were determined. α-fetoprotein (AFP) mRNA (*in situ* hybridisation), cytokeratin-19 and Ki67 antigen expression (immunohistochemistry) and apoptosis (TUNEL) were evaluated on liver tissue samples. Cells with morphological features of oval cells that were cytokeratin-19 (+) and AFP mRNA (+) were scored in morphometric analysis and their proliferation was recorded. In addition, the proliferation and apoptotic rates of hepatocytes were determined.

RESULTS: In the control and sham operated groups, oval cells were significantly less compared to groups Ⅲ, Ⅳ and Ⅴ ($P < 0.001$). The neuropeptides BBS and NT significantly increased the proliferation of oval cells compared to group Ⅲ ($P < 0.001$). In addition, BBS and NT induced a significant increase of hepatocyte proliferation ($P < 0.001$), whereas it decreased their apoptotic activity ($P < 0.001$) compared to group Ⅲ. BBS and NT significantly decreased portal endotoxemia ($P < 0.001$) and increased the hepatic GSH: GSSG ratio ($P < 0.05$ and $P < 0.001$, respectively) compared to group Ⅲ.

CONCLUSION: BBS and NT stimulated oval cell proliferation in a model of liver regeneration, without use of concomitant suppression of hepatocyte proliferation as oval cell activation stimuli, and improved the hepatocyte regenerative response. This peptides-induced combined stimulation of oval cell and hepatocyte proliferation might serve as a possible treatment modality for several liver diseases.

Key words: Liver regeneration; Partial hepatectomy; Hepatic progenitor cells; Oval cells; Apoptosis; Proliferation; Oxidative stress

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INTRODUCTION

The efficiency of the regenerative response of human liver is of major clinical importance for patients' outcome in a number of diverse clinical conditions. When liver damage occurs, it is always followed by regeneration of the organ, which is mainly mediated by proliferation of the non-damaged mature hepatocytes[1,2]. When proliferation of the mature hepatocytes is suppressed, the majority of regeneration is carried out by oval cells, which have the capacity to differentiate into biliary epithelial cells or hepatocytes replacing the lost liver parenchyma[2-5]. Rat oval cells are frequently referred to as equivalent to hepatic progenitor cells in humans[3].

The hepatocyte is the most efficient cell for liver repopulation after injury; however, oval cells participate, possibly as an amplifying transit compartment for hepatocyte differentiation, in processes in which hepatocytes do not respond quickly enough or are unable to respond to proliferative stimuli[6]. Therefore, improving the efficiency of the regenerative response of liver progenitor cells might have a substantial clinical impact, especially in cases of coexisting inhibition of mature hepatocyte proliferation, such as in viral hepatitis[7], chemical toxicity[8] and obstructive cholestasis[9]. For therapeutic application, a non-toxic activation of this stem cell compartment would have been required. Up until now, most experimental trials of pharmaceutical expansion of oval cell compartment have been conducted in models of mature hepatocyte proliferation inhibition[10-12]. Recent studies by our group have demonstrated that oval cells may also proliferate in a model of experimental liver cirrhosis, even in the absence of pretreatment with a known hepatocyte inhibitor[13].

Bombesin (BBS) and neurotensin (NT) are neuropeptides with a wide spectrum of actions on the gut-liver axis, influencing bile acid secretion, enterohepatic circulation, intestinal motility, blood flow, secretion, nutrient absorption and immune response[14-19]. These agents activate diverse intracellular signals in hepatocytes, including induction of mitogenic, antioxidant and metabolic responses[20-23], and confer protection against liver injury and oxidative stress[21,22,24,25]. In our previous studies, we have shown that these neuropeptides reduce hepatic oxidative stress after partial hepatectomy (PHx) and improve the regenerative response of the cholestatic liver in rats[24,26].

This study was undertaken to investigate the possible effectiveness of BBS and NT as a pharmacological intervention for induction of oval cell proliferation in a widely applied experimental model of liver regeneration (PHx) without pretreatment with a known hepatocyte inhibitor.

MATERIALS AND METHODS

Animals

Seventy male albino Wistar rats, weighing 250-320 g, were used in the study. They were housed in stainless-steel cages, three rats per cage, under controlled temperature (23℃) and humidity conditions, with 12 h dark/light cycles, and maintained on standard laboratory diet with tap water ad libitum throughout the experiment, except for an overnight fast before surgery.

The experiments were carried out according to international standards on animal welfare (86/609/EEC) and to the guidelines of the Ethics Committee of Patras University Hospital. The study was approved by the local ethics committee.

Experimental design

Animals were divided randomly into five groups: group Ⅰ (n = 10): non-operated controls; group Ⅱ (n = 15): sham operated; group Ⅲ (n = 15): PHx (70%); group Ⅳ (n = 15): PHx and BBS administration; group Ⅴ (n = 15): PHx and NT administration.

Starting on day 0, the animals of groups Ⅳ and Ⅴ were treated daily with BBS (10 μg/kg, subcutaneously, three times a day) and NT (300 μg/kg, intraperitoneally, once a day) respectively, while the animals of groups Ⅰ, Ⅱ and Ⅲ were divided to receive daily either three subcutaneous or one intraperitoneal injection of 0.5 mL normal saline. Previous studies have shown that the route of saline administration does not affect the results[24]. On the 8th day, animals from groups Ⅲ, Ⅳ and Ⅴ underwent laparotomy and PHx (almost 70%) as described by Higgins and Andersson[27], while animals in group Ⅱ underwent laparotomy and mobilization of the liver. The abdominal incision was closed in two layers with chromic 4-0 cat gut and 4-0 silk. All surgical procedures were performed under strict sterile conditions, using light ether anesthesia. Administration of BBS, NT and normal saline was continued for 48 h after surgery. On the 10th day, all animals were operated (group Ⅰ) or reoperated on (groups Ⅱ, Ⅲ, Ⅳ and Ⅴ), again under strict sterile conditions. Samples were obtained according to the experimental protocol, after which the rats were sacrificed by exsanguination.

Peptides preparation

A stock solution of BBS (Sigma Chemical Co., St. Louis, Missouri, United States) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 1%

(w/v) bovine serum albumin to a concentration of 3.5 μg BBS/0.1 mL. This solution was divided into equal aliquots of 0.1 mL that were stored in plastic tubes at -20℃. At the time of administration, a volume corresponding to a dose of 10 μg BBS/kg body weight was taken from each aliquot and was further diluted with sterile saline to a final volume of 0.5 mL that was injected subcutaneously three times daily. Selection of dose and route of administration was based on previous reports[24].

A stock solution of NT (Sigma Chemical Co., St. Louis, Missouri, United States) was prepared by first dissolving the amount of peptide needed for the study in 1 mL sterile water containing 0.1% (w/v) bovine serum albumin and then diluted with normal saline containing 0.1% (w/v) bovine serum albumin to a concentration of 100 μg NT/0.1 mL. This solution was divided into equal aliquots of 0.1 mL that were stored in glass vials at -20℃. At the time of administration, a volume corresponding to a dose of 300 μg NT/kg body weight was taken from each aliquot and was further diluted with sterile saline to a final volume of 0.5 mL that was injected intraperitoneally once daily. Selection of dose and route of administration was based on previous reports[24].

Portal endotoxin measurements

For the determination of endotoxin concentrations in the portal vein, a laparotomy was performed in all groups, the portal vein was punctured and samples of 1 mL of blood were obtained. Endotoxin concentration was determined by the Limulus Amebocyte Lysate test (LAL, QCL-1000, Lonza, Walkersville, MD, United States) according to the manufacturer's instructions.

Determination of glutathione redox state

After laparotomy, a tissue sample of the liver of each animal was excised, washed in 9 g/L of NaCl and homogenized in sodium phosphate buffer 10 mmol/L, pH = 7.2 (containing 1 mmol/L ethylenediaminetetraacetic acid and 1 mmol/L butylated hydroxyanisole in 0.15% ethanol) by liquid nitrogen for the determination of glutathione redox state. Reduced glutathione was determined spectrophotometrically using Elman's reagent (DTNB) and oxidized glutathione (GSSG) was quantitated by a standard enzymic assay, as described previously[24].

Pathological analysis

***In situ* hybridization for α-fetoprotein expression in paraffin sections:** For the detection of α-fetoprotein (AFP) mRNA (oval cell phenotype), a standard non-radioactive *in situ* hybridization method (ISH) was performed on paraffin sections, as described elsewhere[13]. The Hybridization/Detection Complete System (MBI, Rockville, MD) and the digoxigenin (DIG)-labeled riboprobe for AFP subunit-1 in a 10-fold dilution in hybridization solution were used. Paraffin sections of embryonic rat liver tissue were used as a positive control. To confirm that the positive stain was specific, the slides were processed in an identical way and hybridized with probes known to be complementary to sequences in the test sections (rat genomic DNA probes) (positive control probes). These probes (biotynilated oligonucleotide probes) were similar in length and GC content to AFP probe. For negative control purposes, the slides were processed in the same way but hybridized with heterologous probes. The latter were not complementary to any sequence in the test tissues. These negative control probes were similar in length and GC content to AFP probe.

Immunohistochemistry for the detection of CK19 and Ki67 proteins in paraffin sections: The detection of CK19 protein expression (oval cell phenotype)[5,13] and Ki67 expression (proliferation marker) relied on immunohistochemistry based on a streptavidin biotin peroxidase method (ImmunoCruz™ Staining systems sc-2053; Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, 4-μm thick sections were dewaxed in xylene and hydrated through graded concentrations of alcohol. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 15 min. Sections were then processed in a microwave oven twice for 5 min each time at high power, and subsequently stained with anti-CK19 [goat polyclonal (sc-33119) (Santa Cruz Biotechnology, Santa Cruz, CA) in a dilution of 1:150] and anti-Ki67 [goat polyclonal antibody (M-19) (sc-7846) (Santa Cruz Biotechnology, Santa Cruz, CA) in a dilution of 1:100]. All incubations were performed for 30 min at room temperature. Between the steps, sections were washed in TBS. Diaminobenzidine (Sigma Fast DAB tablets-D-4293, St Louis, MO) was used as the chromogen. Cytoplasmic staining for CK19 and nuclear staining for Ki67 were considered as positive. For negative control purposes, the same streptavidin-biotin technique was used on tissue sections in which 1% BSA in PBS was substituted for the primary antibody.

***In situ* labeling of fragmented DNA for the detection of apoptotic cells:** On paraffin sections, a standard terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labeling (TUNEL) method was employed to detect the fragmented nuclear DNA associated with apoptosis[9]. For this purpose, the *In Situ* Cell Death Detection Kit, POD (Roche, United States) was used according to the manufacturer's instructions. After standard deparaffinization, hydration, incubation with proteinase K and blocking of endogenous peroxidase, tissue sections were incubated: (1) with TdT and DIG-dUTP (TUNEL reaction mixture) at 37℃ for 60 min; and (2) with peroxidase converter anti-fluorescein antibody at 37℃ for 30 min. Diaminobenzidine (Sigma Fast DAB tablets, D-4293, Sigma St. Louis, MO, United States) was used as the chromogen. For physiological positive controls, sections of rat small intestine were subjected to the same procedure. For negative controls, some slides were incubated with label solution that did not contain TdT.

Morphometric analysis: (1) Oval cell measurement: morphometric analysis for the evaluation of oval cell

Table 1 Portal Endotoxin concentrations and hepatic glutathione redox state (mean ± SD)

Markers	Control (Ⅰ) (*n* = 10)	Sham (Ⅱ) (*n* = 15)	PHx (Ⅲ) (*n* = 15)	PHx + BBS (Ⅳ) (*n* = 15)	PHx + NT (Ⅴ) (*n* = 15)
Endotoxin (EU/mL)	0.41 ± 0.07	0.40 ± 0.10	2.45 ± 0.62[a]	1.36 ± 0.53[b]	1.59 ± 0.44[b]
GSH: GSSG	11.55 ± 3.40	12.02 ± 2.85	20.43 ± 4.64[a]	25.88 ± 5.27[c]	28.69 ± 5.94[b]

[a]$P < 0.001$ *vs* sham; [b]$P < 0.001$ *vs* partial hepatectomy (PHx); [c]$P < 0.05$ *vs* PHx. BBS: Bombesin; NT: Neurotensin. GSH: Reduced glutathione hormone; GSSG: Oxidized glutathione.

presence was performed as described previously[13]. Briefly, sections were screened at low power and areas with increased oval cell staining were determined. Cells were scored when they fulfilled the morphological criteria for oval cells (small cells with ovoid nuclei and scant cytoplasm) and expressed AFP mRNA, cytoplasmic protein CK19 and/or nuclear protein Ki67. Cell counts were performed manually at a × 400 magnification (high power field, HPF) using a 10 × 10-microscope grid. The number of oval cells was determined by visual inspection of five non-overlapping different fields per section. The variance in oval cell counts from section to section in the same biopsy was < 10%. The average of these scores was then taken; and (2) Evaluation of proliferation and apoptosis in hepatocytes: immunohistochemical results regarding Ki67 expression and ISH results (TUNEL+ cells) were estimated for hepatocytes. All $Ki67^+$ hepatocytes were considered proliferating cells. Regarding apoptosis, in order to avoid its overestimation by the TUNEL method, hepatocytes were considered apoptotic only if, in addition to positive TUNEL stain, they displayed the morphological features of apoptosis on light microscopy (cytoplasmic fragmentation and nuclear condensation) and were not "surrounded" by inflammatory elements. Estimation of proliferation and apoptosis in each case was performed by a stereological method. Specifically, sections from each liver biopsy were viewed through a light microscope with × 40 flat field objective. A square lattice of 100 points with a total surface area of 0.064 mm^2 was superimposed onto the tissue. Data were collected from a series of randomly selected 15 adjacent fields extending throughout the biopsy. For each field a percentage value for each parameter ($Ki67^+$ cells, apoptotic cells) was obtained by dividing the points falling on stained tissue by the total number of measured points. Also, for each field, the ratio of the obtained values (% $Ki67^+$ cells/% apoptotic cells) was calculated as a balancing index expressing net cell turnover. It should be noted that the variance in cell counts from field to field in the same section was < 10%. The average of these scores was then taken and expressed as proliferation and apoptotic indexes and proliferation/apoptosis ratio respectively for each case.

Statistical analysis

Data were analyzed using the SPSS statistical package (SPSS Inc., 2001, Release 11.0.0, United States). In groups Ⅰ, Ⅱ and Ⅲ, data obtained from subcutaneously and intraperitoneally saline-treated rats were pooled, as there was no significant difference between differentially injected animals for all parameters studied. Results are expressed as mean (SD). Comparisons among multiple groups were performed using the one-way ANOVA, followed by Bonferroni's post hoc test when variances across groups were equal or by Dunnette's T3 post hoc test when variances were not equal. Variance equality was tested by Levene statistical analysis. In all cases, differences were considered significant when $P < 0.05$.

RESULTS

Portal endotoxin concentration

Hepatectomized animals (group Ⅲ) presented significantly elevated endotoxin concentrations in portal blood compared with groups Ⅰ and Ⅱ ($P < 0.001$). Treatment with BBS or NT led to significantly lower endotoxin values in portal vein in groups Ⅳ and Ⅴ ($P < 0.001$ *vs* group Ⅲ, respectively) (Table 1).

Hepatic glutathione redox state

Evaluation of glutathione redox state showed significantly increased levels of reduced glutathione hormone (GSH):GSSG in hepatectomized rats of group Ⅲ ($P < 0.001$ *vs* groups Ⅰ and Ⅱ). Administration of BBS or NT resulted in further increase of GSH:GSSG ratio in groups Ⅳ and Ⅴ ($P < 0.05$ and $P < 0.001$ *vs* group Ⅲ, respectively) (Table 1).

Oval cell detection and proliferation

Oval cells were present in all specimens studied. In the control and sham operated groups, oval cells were significantly less compared to groups Ⅲ, Ⅳ and Ⅴ ($P < 0.001$, Table 2). In PHx rats (group Ⅲ) they were located in periportal areas and the formation of small ducts was occasionally recorded. Oval cells expressed CK19 (Figure 1A and B), AFP mRNA (Figure 1C and D) and Ki67. When rats subjected to PHx were treated with either BBS (group Ⅳ) or NT (group Ⅴ), the levels of expression of all three molecules were significantly increased compared to group Ⅲ ($P < 0.001$, Table 2).

Proliferation and apoptosis detection in hepatocytes

The proliferation index of hepatocytes was significantly higher in group Ⅲ as compared to groups Ⅰ and Ⅱ ($P < 0.001$, respectively). Administration of BBS or NT in PHx rats induced a significant increase of hepatocyte proliferation in groups Ⅳ and Ⅴ compared to group Ⅲ ($P < 0.001$, respectively, Table 3) (Figure 2A and B).

Table 2 Morphometric analysis of oval cell presence and proliferation (marker-positive oval cells per high power field) (mean ± SD)

Markers	Control (Ⅰ) (*n* = 10)	Sham (Ⅱ) (*n* = 15)	PHx (Ⅲ) (*n* = 15)	PHx + BBS (Ⅳ) (*n* = 15)	PHx + NT (Ⅴ) (*n* = 15)
CK19 protein	1.65 ± 0.31	1.63 ± 0.28	6.22 ± 0.82[a]	18.51 ± 2.31[b]	19.37 ± 3.48[b]
AFP mRNA	1.34 ± 0.25	1.31 ± 0.21	5.45 ± 0.91[a]	16.32 ± 1.81[b]	17.53 ± 4.12[b]
Ki67 protein	1.28 ± 0.14	1.26 ± 0.11	5.22 ± 0.11[a]	15.61 ± 2.54[b]	16.64 ± 3.59[b]

[a]$P < 0.001$ *vs* sham; [b]$P < 0.001$ *vs* partial hepatectomy (PHx). BBS: Bombesin; NT: Neurotensin; AFP: α-fetoprotein.

Table 3 Hepatocytes' proliferation and apoptosis (mean ± SD)

Markers	Control (Ⅰ) (*n* = 10)	Sham (Ⅱ) (*n* = 15)	PHx (Ⅲ) (*n* = 15)	PHx + BBS (Ⅳ) (*n* = 15)	PHx + NT (Ⅴ) (*n* = 15)
Proliferation index	5.95 ± 1.33	6.12 ± 1.64	18.32 ± 3.11[a]	27.25 ± 4.13[b]	25.62 ± 3.41[b]
Apoptotic index	0	0	19.31 ± 4.16[a]	9.24 ± 2.65[b]	8.97 ± 4.14[b]
Proliferation/apoptosis	-	-	0.94 ± 0.74	2.99 ± 1.55[b]	2.85 ± 0.82[b]

[a]$P < 0.001$ *vs* sham; [b]$P < 0.001$ *vs* partial hepatectomy (PHx). BBS: Bombesin; NT: Neurotensin.

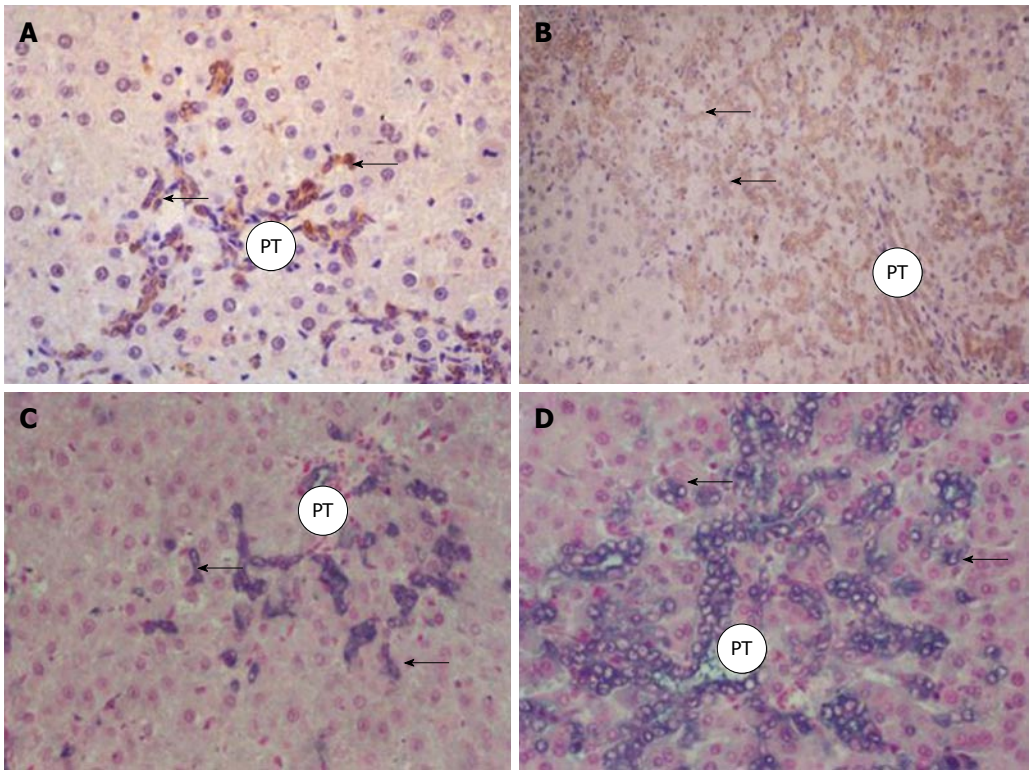

Figure 1 Microphotographs showing expression of CK19 from oval cells (arrows) in livers of group Ⅲ (A) and group Ⅳ (B) and α-fetoprotein mRNA expression from oval cells (arrows) in group Ⅲ (C) and group Ⅳ (D). In group Ⅳ there is higher oval cell presence as demonstrated by both markers [CK19: streptavidin biotin peroxidase (A) × 250, (B) × 100, α-fetoprotein mRNA: *in situ* hybridization × 250]. PT: Portal tract.

In control and sham operated rats, no apoptotic bodies were detected in hepatocytes. After PHx (group Ⅲ), increased apoptotic activity was detected in lobules, whilst administration of BBS or NT significantly decreased the apoptotic index ($P < 0.001$, for groups IV and V compared with group Ⅲ, Table 3) (Figure 2C and D). The proliferation/apoptosis ratio was significantly increased in groups Ⅳ and Ⅴ compared to group Ⅲ ($P < 0.001$, respectively).

DISCUSSION

Effective liver regeneration after extended liver resection or hepatocytic necrosis is of great clinical importance and several experimental studies have focused on the pharmacological augmentation of these process. Experi-

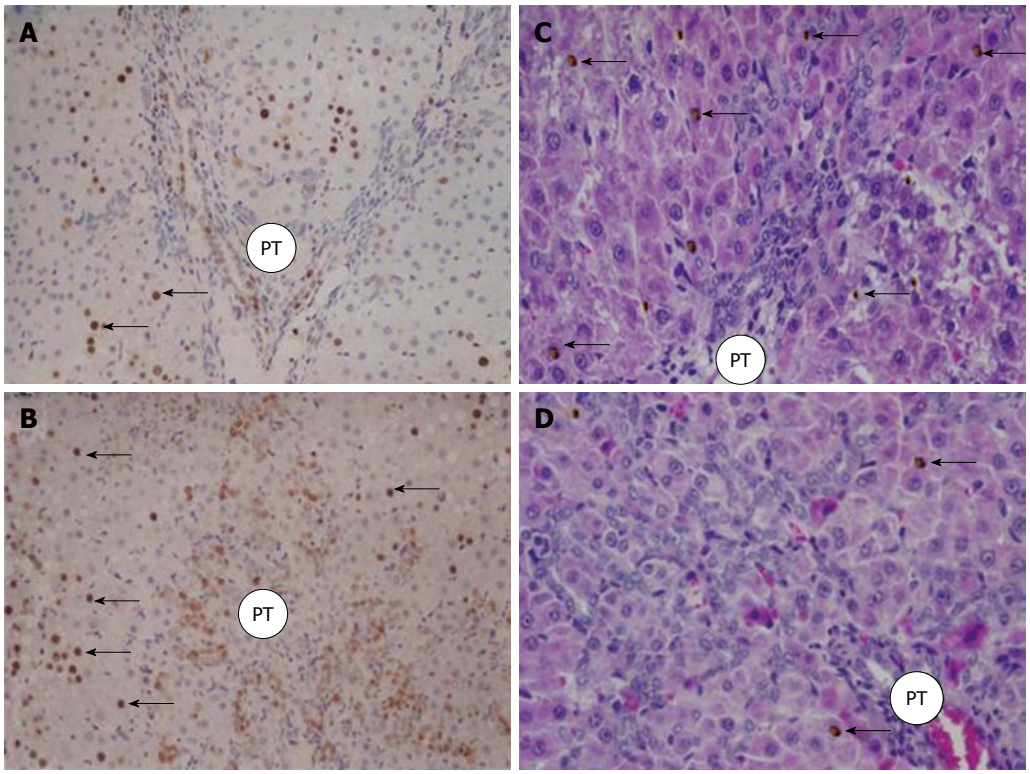

Figure 2 Microphotographs showing Ki67 (+) hepatocytes (arrows) from livers of group Ⅲ (A) and group Ⅳ (B) and TUNEL (+) hepatocytes (arrows) in group Ⅲ (C) and group Ⅳ (D). In group IV there are more Ki67 (+) hepatocytes (streptavidin biotin peroxidase × 100) and less TUNEL (+) hepatocytes (streptavidin biotin peroxidase and hematoxylin eosin × 250). PT: Portal tract.

mentally, the PHx and the carbon tetrachloride administration animal models have been widely used to simulate the clinical conditions of liver resection or hepatocytic necrosis, respectively. Previous studies have unequivocally shown that the hepatocytes are the replicating cells responsible for liver regeneration in these models and that progenitor cell activation leading to lineage generation is not observed during these processes[28]. Despite the low replicative rate of hepatocytes in the normal liver, these highly differentiated cells are not terminally differentiated and replicate in a highly regulated manner after loss of cell or tissue mass[28]. On the other hand, oval cells constitute a transit amplifying cell compartment, which is activated when hepatocyte proliferation is compromised[5,29]. The biological effect of several primary hepatocyte mitogens on hepatocytic proliferation has been extensively studied in experimental models but there are hardly any data about the influence of these compounds on oval cells[10,30,31]. Moreover, to the best of our knowledge, all *in vivo* pharmaceutical trials of oval cell compartment expansion have been conducted in conditions of mature hepatocyte proliferation inhibition, which does not allow safe conclusions on the clear effect of tested compounds on oval cell proliferation and also does not always simulate the clinical situation.

The present study evaluated the effect of the neuropeptides BBS and NT on oval cells and hepatocytes in partially hepatectomized rats, a widely applied model of liver regeneration. The experimental design did not include any treatment with known inhibitors of hepatocyte proliferation as a method for oval cell compartment activation. Therefore, we aimed at evaluating *in vivo* the potential net proliferative effect of tested peptides on oval cells for the first time. The results presented clearly demonstrate the proliferative effect of BBS and NT on oval cells in partially hepatectomized rats in conjunction with improvement of the regenerative response of mature hepatocytes evidenced by promotion of hepatocytes' proliferation and prevention of their apoptosis. Promotion of oval cell proliferation is of great clinical importance profoundly in cases of inhibition of hepatocyte proliferation as this cell type carries on the process of liver repopulation, as well as in cases of effective hepatocyte proliferation, providing a ready cell compartment to continue liver regeneration if a hepatocytic inhibitory insult arises. The presence of hepatic progenitor cells in failed livers at autopsy and in livers removed at transplantation indicates the importance of the efficiency of their regenerative response[32]. Agents that stimulate oval cell proliferation and differentiation, or transplantation of oval cells could be a potent therapeutic modality in the treatment of patients with fatal liver disease, such as fulminant hepatic failure.

In our previous studies with experimentally jaundiced rats, we have shown that BBS and NT attenuated oval cell proliferation in the cholestatic liver, inducing cell type-specific effects on oval cells, hepatocytes and cholangiocytes[26]. The findings of the present study with

promotion of oval cell proliferation suggest that oval cells' response to BBS and NT may be different depending on the type of liver injury. Taking into consideration our previous and current findings, one should assume that neuropeptides' effects on oval cells may be significant for the regeneration of a healthy liver (e.g. in living donor transplantation), but not relevant in an injured liver. However, in our previous experiments with bile duct ligated rats, BBS and NT significantly improved cholestatic liver injury, despite reduction of oval cell proliferation[26]. This finding might reflect the fact that induction of hepatocytes' regenerative response by neuropeptides' action is effective for liver repair in cholestasis, diminishing the role of oval cell participation in this process. On the other hand, in the model of liver regeneration used in the present study, the induction of hepatocytes' regenerative response by BBS and NT might be relatively insufficient for an effective liver repopulation, thus activating the transit amplifying compartment of oval cells.

The biological effects of BBS and NT on hepatic oval cells of rats subjected to PHx may result from direct receptor-mediated action[33,34]. Specific G protein-coupled receptors of BBS and NT have been previously identified in hepatocytes and cholangiocytes[33-35]. Similarly G protein-coupled receptors have been identified in oval cells as well, and their expression has been interrelated with the activation with this stem cell compartment[36]. The growth pathways that govern activation and liver differentiation of liver stem cells after PHx are quite complex and not fully elucidated, involving interplay of diverse cytokines and growth factors such as the hepatocyte growth factor (HGF) and the transforming growth factor (TGF)-β[37]. Although a very rapid increase in tumor necrosis factor-α levels after hepatectomy (possibly endotoxin-induced) is considered as the first step in activation of these growth factors[38], reduction of portal endotoxemia by BBS and NT, demonstrated in the present study, does not preclude the activation of oval cells *via* diverse HGF and TGF dependent pathways. In addition, an indirect mechanism of action of BBS and NT on oval cells in the regenerating liver could exist through the action of other gastrointestinal or systemic hormones released in response to these neuropeptides[39-44].

The present study also demonstrated that BBS and NT enhanced hepatocytes' regenerative response, attributed to increased proliferation and decreased apoptosis of mature hepatocytes and theoretically to increased transition of oval cells to mature hepatocytes. Estimation of hepatocytes' proliferation/apoptosis ratio showed that BBS and NT induced a threefold net increase in proliferating over apoptotic hepatocytes in the regenerating liver. Hepatocytes' apoptosis, which is a major factor of a defective regenerative response, could have been attenuated by neuropeptides administration, either through a direct receptor-mediated mechanism or indirectly through reduction of hepatic oxidative stress and portal endotoxemia shown in the present study, with mechanisms previously reported[24,45]. According to the present and our previous results, liver regeneration takes place under low oxidative stress conditions; however, the further attenuation of hepatic oxidative stress induced by BBS and NT might contribute to the augmentation of the hepatocytes' regenerative response[24].

In conclusion, the present study demonstrates that the neuropeptides BBS and NT exert a net proliferative effect on oval cells in a model of liver regeneration without use of concomitant suppression of hepatocyte proliferation as oval cell activation stimuli. Concurrently, these factors promote hepatocyte proliferation and prevent its apoptosis, thus improving the hepatic regenerative response. Although the results from animal studies should be transferred with much caution in clinical practice, we feel that there is an emerging need for further evaluation of our findings, as the observed pharmacological combined stimulation of hepatocyte and oval cell proliferation might serve as a possible treatment modality for several liver diseases.

COMMENTS

Background

The regenerative response of human liver is of major clinical importance for patients' outcome in a number of diverse clinical conditions. The hepatocyte is the most efficient cell for liver repopulation after injury; however, oval cells participate, possibly as an amplifying transit compartment for hepatocyte differentiation, in processes in which hepatocytes do not respond quickly enough or are unable to respond to proliferative stimuli.

Research frontiers

Pharmacological augmentation of the hepatic regenerative response in diverse types of liver injury has been the topic of intense research for several decades. Improving the efficiency of the regenerative response of liver progenitor cells might have a substantial clinical impact, especially in cases of coexisting inhibition of mature hepatocyte proliferation, such as in viral hepatitis, chemical toxicity and obstructive cholestasis. Up until now, most experimental trials of pharmaceutical expansion of oval cell compartment have been conducted in animal models of mature hepatocyte proliferation inhibition by toxic chemical compounds. However, for therapeutic application a non-toxic activation of this stem cell compartment is required.

Innovations and breakthroughs

The present study evaluated the effect of the neuropeptides bombesin (BBS) and neurotensin (NT) on oval cells and hepatocytes in partially hepatectomized rats, a widely applied model of liver regeneration. The experimental design did not include any treatment with known inhibitors of hepatocyte proliferation as a method for oval cell compartment activation. Therefore, we aimed at evaluating *in vivo* the potential net proliferative effect of the tested peptides on oval cells for the first time. The results presented clearly demonstrate the proliferative effect of BBS and NT on oval cells in partially hepatectomized rats in conjunction with improvement of the regenerative response of mature hepatocytes, evidenced by promotion of hepatocytes' proliferation and prevention of their apoptosis.

Applications

Promotion of oval cell proliferation is of great clinical importance profoundly in cases of inhibition of hepatocyte proliferation as this cell type carries on the process of liver repopulation, as well as in cases of effective hepatocyte proliferation, providing a ready cell compartment to continue liver regeneration if a hepatocytic inhibitory insult arises. BBS and NT promoting oval cell and hepatocyte proliferation could be a potent therapeutic modality in the treatment of patients with several liver diseases, such as fulminant hepatic failure.

Peer review

This is an interesting observational study. The concept is new, the results are robust and may provide potential target for clinical patient care.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

March 03-05, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 07-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 06-07, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 07-09, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 07-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 01-04, 2011
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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Patent (list all authors)

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

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