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Ultrastructure of intestinal mucosa in diarrhea-predominant irritable bowel syndrome

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Objectives: Impaired intestinal barrier function has been demonstrated in the pathophysiology of diarrhea-predominant irritable bowel syndrome (IBS-D). This study aimed to describe the intestinal ultrastructural findings in the intestinal mucosal layer of IBS-D patients. *Methods:* In total, 10 healthy controls and 10 IBS-D patients were analyzed in this study. The mucosa of each patient's rectosigmoid colon was first assessed by confocal laser endomicroscopy (CLE); next, biopsied specimens of these sites were obtained. Intestinal tissues of IBS-D patients and healthy volunteers were examined to observe cellular changes by transmission electron microscopy (TEM). *Results:* CLE showed no visible epithelial damage or inflammatory changes in the colonic mucosa of IBS-D compared with healthy volunteers. On transmission electron microscopic examination, patients with IBS-D displayed a larger apical intercellular distance with a higher proportion of dilated (>20 nm) intercellular junctional complexes, which was indicative of impaired mucosal integrity. In addition, microvillus exfoliation, extracellular vesicle as well as increased presence of multivesicular bodies were visible in IBS-D patients. Single epithelial cells appeared necrotic, as characterized by cytoplasmic vacuolization, cytoplasmic swelling, and presence of autolysosome. A significant association between bowel habit, frequency of abdominal pain, and enlarged intercellular distance was found. *Conclusion:* This study showed ultrastructural alterations in the architecture of intestinal epithelial cells and intercellular junctional complexes in IBS-D patients, potentially representing a pathophysiological mechanism in IBS-D.

Keywords: diarrhea-predominant irritable bowel syndrome, intercellular junctional complex, transmission electron microscopy, extracellular vesicles, intestinal barrier

Introduction

Irritable bowel syndrome (IBS) is a common and economically important gastrointestinal (GI) disorder that is characterized by abdominal pain/discomfort and changes in bowel habits (18); in China, IBS affects 2.9%–15.6% of the general population, depending on the diagnostic criteria that are used (4, 10). IBS is classified as the following subtypes: predominant stool pattern into diarrhea-predominant (IBS-D), constipation-predominant, mixed, and unclassified. It has been reported that IBS-D is commonly encountered in China (17) and causes considerable healthcare costs; it also impairs work productivity and quality of life.

Although the exact pathogenic mechanisms of IBS-D remain largely unknown, studies have provided evidence for the presence of altered intestinal epithelial barrier in the colonic

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mucosa, challenging the classical conception of IBS-D as a functional disorder (21, 23). Several studies indicated that abnormal intestinal barrier function might play a vital role, because it enables increased antigenic penetration, leading to activation of mucosal immune responses and contributing to the generation and persistence of GI symptoms (12). Thus, deciphering the pathophysiological mechanisms underlying IBS-D is a major public health-care priority in China.

The intestinal epithelial barrier acts as the first boundary of defense to prevent the passage of harmful intraluminal entities, including foreign antigens, microorganisms, and their toxins. From a structural perspective, these functions are preserved by a number of features, including a monolayer of epithelial cells and intercellular junctional complexes that can be identified at the ultrastructural level: desmosomes, adherens junctions (AJs), and tight junctions (TJs) (8). To date, studies investigating intestinal barrier abnormalities have largely focused on the molecular and cellular mechanisms of TJ dysfunction in IBS-D (23). Indeed, several findings indicated that mucosal barrier defects in IBS-D patients were not limited to the TJ (21). Defects in the integrity of the AJ and desmosomes have been proposed to underlie the increased mucosal permeability in GI disease, such as functional dyspepsia (28) and inflammatory bowel disease (2). To date, there have been few studies that have focused on the ultrastructural changes of AJ and desmosomes in IBS-D patients. Furthermore, there is mounting evidence to indicate that abnormalities in the intestinal epithelial cells are involved in the disruption of the intestinal barrier (9, 20). Thus, the general hypothesis of our research program is that IBS-D is a disorder that primarily affects not only intercellular junctional complexes but also the architecture of epithelial cells in the intestinal mucosa.

The electron microscope technique stands out for its distinct abilities to assess the relationship of the intestinal barrier within the context of cell structure and to enable the direct observation of structures whose existence could only be inferred by other methods. Thus, we used the technology of transmission electron microscopy (TEM) to examine the architecture of the intestinal epithelium in IBS-D patients and healthy controls. The purpose of this study was to illustrate alterations of the intestinal barrier in IBS-D and to describe ultrastructural changes in the intestinal mucosal layer by confocal laser endomicroscopy (CLE) and TEM.

Methods

Patients

Patients (5 men and 5 women; age = 50.0 ± 14.1 years) referred for the evaluation of IBS symptoms who presented daily watery diarrhea or mushy stools and who met the Rome III IBS-D criteria were recruited at the outpatient clinic of the Department of Gastroenterology at the Qilu Hospital of Shandong University. Healthy subjects (5 men and 5 women; age = 44.8 ± 9.4 years) underwent endoscopy for polyps and cancer screening and had negative results. A complete history and physical examination were performed in all subjects. Organic GI disorders were excluded by routine laboratory tests and endoscopies with biopsies. Other exclusion criteria for all subjects were the following: intake of non-steroidal anti-inflammatory drugs, probiotics, histamine antagonists, corticosteroids, or other immunosuppressive drugs in the preceding 6 months, diabetes or celiac disease, pregnancy or breast-feeding, allergic diseases and psychiatric disorders, abdominal surgery history, personal history of inflammatory bowel disease and digestive cancer, or previous history of acute gastroenteritis in relation to the initiation of IBS symptoms. A complete colonoscopy was performed by an experienced

endoscopist after routine bowel preparation with 4 L of macrogol solution. During the colonoscopies, biopsies were taken in the rectosigmoid colon in all cases. This study was approved by the clinical ethical committee of Qilu Hospital of Shandong University, and all participants gave their written informed consent before participation.

Clinical assessment

IBS-D patients were asked to record the frequency of defecation (day with maximum number of bowel movements) and stool consistency assessed by the Bristol Stool Form Score (16). In addition, patients were asked to score the frequency of their abdominal symptoms over the past 2 weeks using a validated questionnaire.

Confocal laser endomicroscopy (CLE)

Before the CLE (EC3870KPentax, Tokyo, Japan) examination, patients had standard cardiopulmonary monitoring and received intravenous sedation with midazolam and fentanyl. After intubation of the terminal ileum, 5 ml of 10% fluorescein solution was administered intravenously. CLE examination was performed by two experienced endoscopists in real time in each case. At least five different sites within a 10-cm radius in the rectosigmoid were imaged in all patients. To minimize bias, images were analyzed after examination by two reviewers who were blinded to the status of the patients and the indication for the colonoscopy.

Transmission electron microscopy (TEM)

Rectosigmoid biopsies for TEM were cut into 1-mm3 small squares and immediately fixed in cacodylate-buffered 2.5% glutaraldehyde solution at 4 °C overnight, washed in cacodylate buffer (0.1 mol/L), and post-fixed with 1% osmiumtetroxide for 1 h at room temperature. After washing in cacodylate buffer (0.1 mol/L), samples were dehydrated in graded concentrations of acetone and embedded in araldite. Samples were cut into semithin sections (500 nm) with an ultramicrotome equipped with a glass knife, whereas ultrathin sections (70 nm) were cut on the same microtome equipped with a diamond knife. Toluidine blue-stained semithin sections were screened under an optical microscope to observe colonic epithelial layers. Following this step, ultrathin sections were double stained with uranyl acetate and lead citrate and observed under a JEOL CX1200 electron microscope (Beijing, China). To evaluate changes in intercellular junctional complex morphology, the junctional regions of two randomly chosen villi were examined in each specimen. Examinations were performed on a total of approximately 30 intercellular junctional complexes per specimen in non-overlapping fields. Intercellular junctional complexes were considered open when the size of dilatation was more than 20 nm (5).The number of multivesicular bodies apical to the surface of the epithelial cell was counted in a total of 30 sections per specimen in well-oriented villi. Data on experiments and diagnosis were blinded to the examiners.

Statistical analysis

All values were expressed as means ± SD. Statistical analysis was analyzed using SPSS 20.0 (SPSS, Chicago, IL, USA); data were considered to be statistically significant when $p < 0.05$. Significant differences between IBS-D patients and healthy controls were evaluated by a two-tailed Student's *t*-test. Relationships between clinical variables and ultrastructural alterations were assessed by Spearman's ρ correlation in IBS-D patients.

Results

Group characteristics

The clinical characteristics of control subjects and patients with IBS-D are described in Table I. No significant differences in age were detected between IBS-D patients and healthy subjects ($p = 0.12$). The colonic mucosa was macroscopically normal in all subjects.

Confocal laser endomicroscopy (CLE)

Representative CLE images from control and IBS-D patients are shown in Fig. 1. Endomicroscopy revealed no differences in epithelial architecture between IBS-D patients and healthy subjects. Using CLE, we demonstrated round and regularly arranged crypts and intact epithelial integrity in the colonic epithelia of IBS-D patients and healthy subjects. Although fluorescein entered the lateral intercellular spaces up to the apical border, there was no escape into the lumen in IBS-D patients.

Ultrastructural observation of intestinal epithelial cells in healthy controls

TEM analysis of the intestinal epithelial mucosa in healthy controls showed polarized cells and the presence of cell–cell junctional complexes between individual cells, demonstrating the ability of intestinal epithelial cells to support the structural integrity of the epithelial barrier. As shown in Fig. 2, TJ sealed the space between adjacent epithelial cells near the apical surface. Below the TJ, intercellular junctional complexes were subsequently formed by the AJ, desmosomes, and intercellular folds from top to bottom. AJ formed electron-dense structures between membranes of adjacent cells that were located beneath the TJ. Desmosomes were identified and quantified as discrete, linear hyperdensities along the outer cellular membrane. They could be distinguished from AJ on the basis of their attachment to intermediate filaments. The cytoplasm contained numerous large oval- or rod-like mitochondria; some of these were coupled with the vesicular structure containing low-density material.

Ultrastructural alterations in IBS-D patients

IBS-D patients exhibited a larger apical intercellular distance with a higher percentage of dilated (>20 nm) intercellular junctions compared with healthy controls (18.22% ± 5.32% vs.

Table I. Clinical characteristics of the study population

	HC ($n = 10$)	**IBS-D ($n = 10$)**	***p* value**
Age (years)	44.8 (9.4)	50.0 (14.1)	0.34
Sex (F/M)	5/5	5/5	–
IBS duration (years)	0	6.8 (4.5)	–
Frequency of abdominal pain/discomfort	0	2.2 (0.9)	–
Bowel movements	1.0 (0.5)	3.4 (0.5)	<0.001
Stool form, Bristol score	3.8 (0.6)	5.9 (0.6)	<0.001

Quantitative data are expressed as the mean (SD) or median (range). Grading standard in the Bristol Stool Form Scale is used to record stool characteristics of patients with IBS-D. HC: healthy controls; IBS: irritable bowel syndrome; IBS-D: diarrhea-predominant IBS; F: female; M: male

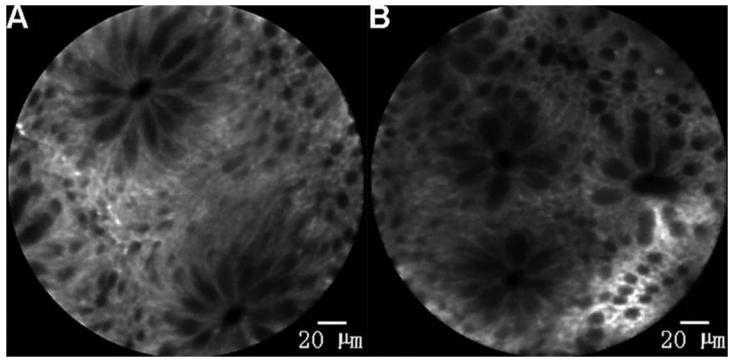

Fig. 1. Representative confocal laser endomicroscopy images of rectosigmoid mucosa in healthy controls (A) and IBS-D patients (B). Scale bar: 20 μm

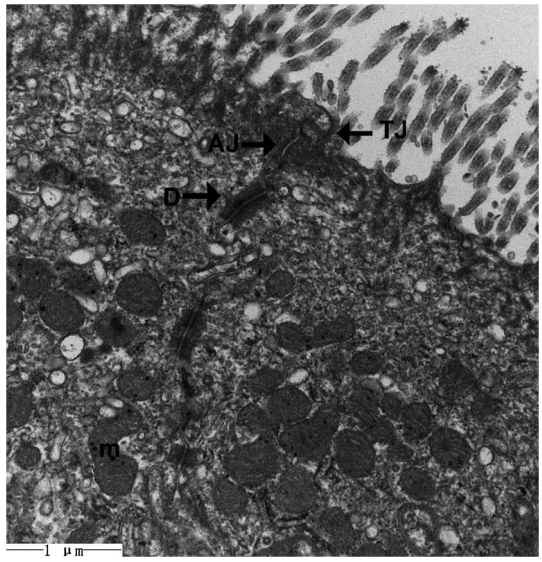

Fig. 2. Ultrastructure features of intercellular junctional complex in the colonic mucosa of healthy controls. Intact intercellular junctional complexes were formed by the TJ, AJ, and desmosomes from top to bottom. TJ: tight junction; AJ: adherens junction; MV: microvillus; m: mitochondria. Scale bar: 1 μm

27.79% ± 7.92%, $p = 0.005$; Fig. 3B). In Fig. 3A, the ultrastructure of TJ featured real disassembly along with dilatation and destruction of AJ and desmosomes.

The less electron-dense cytoplasm of epithelial cells contained a number of swollen mitochondria and multivesicular bodies (Fig. 3C), which were characterized by the presence of distinctive intraluminal vesicles. The mitochondrial matrix was lucent, and the cristae were dilated (Figs 3A and 4A). Indeed, IBS-D patients showed increased multivesicular body counts compared with healthy controls (6.6 ± 4.27 vs. 13.5 ± 7.59, $p = 0.02$; Fig. 3D).

In IBS-D patients, we also noticed significant changes of microvilli in the apical plasma membrane compared to healthy controls, showing exfoliated and incomplete microvilli, microvillus vesiculation, the presence of microvilli-forming vesicles (Fig. 4A), and extracellular vesicles (Fig. 4B). Several epithelial cells had sparsely distributed microvilli (Fig. 3C).

Additionally, there were several individual cells exhibiting signs of necrosis. Specifically, these cells showed extensive vacuole formation (Fig. 5B), cytoplasmic swelling, cell organelle extrusion into the lumen, the presence of a double membrane structure resembling autolysosome, and internal lysis of all organelles and the entire cytoplasm (Fig. 5A).

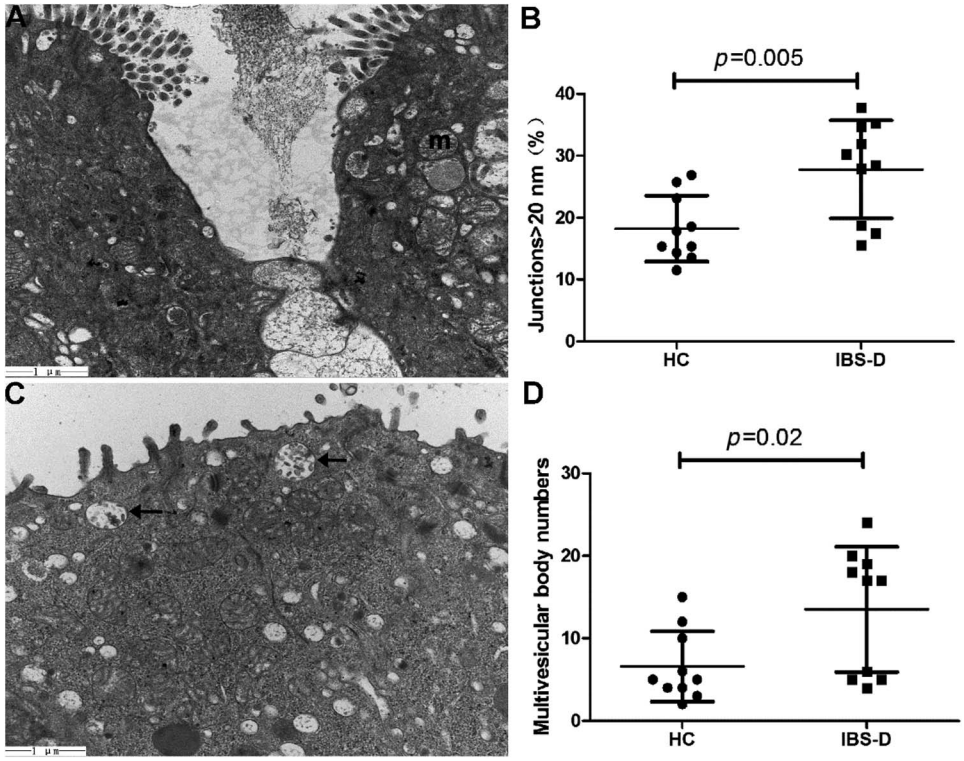

Fig. 3. Ultrastructure of intercellular junctional complex and multivesicular body in the colonic mucosa of IBS-D patients. (A) Intercellular junctional complexes were damaged with widened intercellular spaces. (B) IBS-D patients had a higher percentage of dilated (>20 nm) intercellular junctions compared with healthy controls. Some mitochondria appeared ruptured or vacuolized. (C) Multivesicular bodies were present (arrow) in the cytoplasm and microvilli were sparsely distributed at the apical plasma membrane. (D) IBS-D patients showed increased multivesicular body counts compared with healthy controls. m: mitochondria; HC: healthy controls; IBS-D: diarrhea-predominant IBS. Scale bar: 1 μm

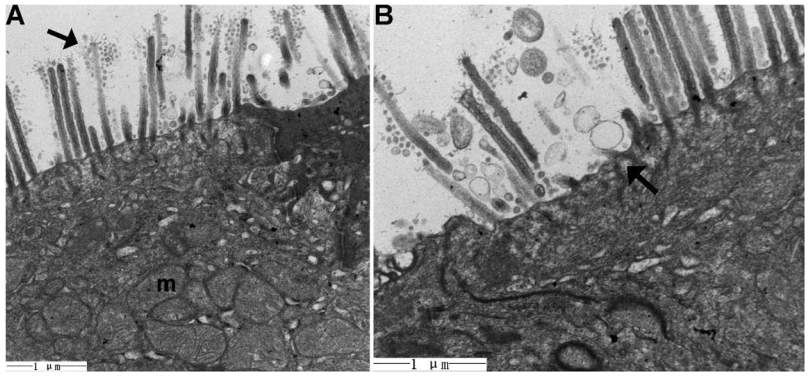

Fig. 4. Ultrastructural changes on the surface of colonic epithelial cells in IBS-D patients. (A) The apical plasma membrane showed incomplete microvilli, microvillus vesiculation, and the presence of microvilli-forming vesicles (arrow). (B) Increased extracellular vesicles shed from the surface of intestinal epithelial cells (arrow). m: mitochondria. Scale bar: 1 μm

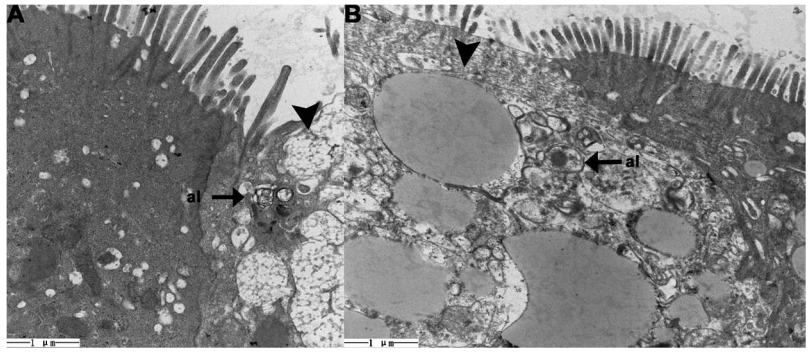

Fig. 5. Ultrastructural features of necrotic cells in the intestinal mucosa of IBS-D patients. A few epithelial cells appeared necrotic, as characterized by the presence of autolysosomes (arrows in A and B), cytoplasmic lucency and vacuolization (arrowhead in B), and severely dilated and lytic cell organelles (arrowhead in A). al: autolysosomes. Scale bar: 1 μm

Correlation of ultrastructural alterations with clinical manifestations

The association between ultrastructural alterations and several clinical factors in the patient group was evaluated. Ultrastructural junction impairment exhibited good correlation with bowel movement ($r = 0.78$, $p = 0.009$) and stool consistency ($r = 0.74$, $p = 0.01$). A significant positive relationship was found between the frequency of abdominal pain and enlarged intercellular distance ($r = 0.85$, $p = 0.002$). However, there was no correlation between multivesicular body counts with clinical factors ($p > 0.05$ for all).

Discussion

Although CLE imaging did not exhibit mucosal abnormalities in the colonic epithelium of IBS-D patients, examination of the mucosa by TEM demonstrated ultrastructural alterations in the architecture of intestinal epithelial cells and intercellular junctional complexes compared with healthy volunteers. Indeed, patients with IBS-D were characterized by dilated intercellular spaces, providing support for barrier defects in the pathogenesis of IBS-D. Nevertheless, neither several of the ultrastructural changes, including the presence of extracellular vesicles in the epithelial surface and multivesicular bodies in the cytoplasm nor necrotic cell death has been described in any previous studies to the best of our knowledge.

The optical biopsy instrument CLE, a newly invented diagnostic tool, provides real-time optical section images with a cellular resolution similar to that of histology (13). The fact that CLE is combined with routine conventional colonoscopy makes it an attractive and convenient diagnostic methodology for routine clinical use. Gut mucosa changes and intestinal permeability can be made visible using CLE (7, 14). Recently, CLE enabled visualization of altered duodenal and ileum mucosal barrier function in patients with IBS (7, 27). To date, there have been few CLE studies that have focused on the structure of the large intestine in patients with IBS-D. However, our CLE study showed no visible epithelial damage or inflammatory change in IBS-D intestines, potentially indicating that CLE had low sensitivity and specificity in the detection of colonic epithelial lesions in IBS-D. The other possible explanation for this phenomenon might be the small sample size in this study.

In this study, the healthy group displayed integrity of the mucosa barrier with a cellular ultrastructure in the colonic epithelium, whereas profound ultrastructural disruption was observed in the intercellular junctional complexes of IBS-D patients with widened TJ, disruption of AJ, and desmosomes. The significant association between dilated intercellular space and clinical parameters supports the hypothesis that the disruption of intercellular junctional complexes may play a vital role in IBS-D pathophysiology, and these changes may be responsible for diarrhea in these patients.

It is well-known that homeostatic intestinal barrier function is regulated by intercellular junctional complexes, in which TJs are the most important constituents (15). A TJ is a multiprotein complex that links adjacent cells, regulates transport through the extracellular matrix, and allows limited passage of larger molecules. Structurally, the TJ is composed of membrane-spanning proteins, including occludin, zonaoccludens, and claudins. Many previous studies have demonstrated decreased expression of TJ proteins in the intestinal mucosa of IBS-D patients (1, 21). It is noteworthy that we detected structural disruptions in AJ and desmosomes. A number of studies have reported that AJs are important not only for promoting cell–cell adhesion but also for maintaining cell polarity, affecting the formation of other adhesive complexes, such as desmosomes and regulating apoptosis, migration and proliferation of epithelial cells (11, 22). Desmosomes are considered to be involved in the mechanical linkage of adjacent cells (11). This finding raises the possibility that AJ and desmosomes dysregulation is not only involved in the impairment of epithelial barrier function but also has other effects on cell proliferation and apoptosis in IBS-D. However, we did not examine the expression of AJ proteins and desmosomal proteins in IBS-D. Further studies are required to delineate the role of these proteins in the maintenance of homeostatic intestinal barrier function and epithelial architecture in patients suffering from IBS-D.

We observed significant changes on the surface of epithelial cells, including microvillus exfoliation, microvillus vesiculation, and extracellular vesicle formation. This is the first study to demonstrate the presence of increased extracellular vesicles shed from the surface of intestinal epithelial cells in patients with IBS-D (Fig. 4). These vesicles are apparently nipped off into the environment. Two distinct types of extracellular vesicles were observed in the intestinal mucosa: (1) the larger size (referred to as “microvesicles” or “shed microvesicles”) is heterogeneous (50–1,500 nm) and considered to bud directly from the plasma membrane; (2) the smaller size class (referred to as “exosomes”) is relatively homogeneous in size (50–120 nm), has endocytic origins, and is released by multivesicular bodies (29). In this study, it was possible to observe the increased presence of multivesicular bodies (Fig. 3C) that were rich in distinctive intraluminal vesicles. It had been proposed that multivesicular bodies fused with the plasma membrane and released their vesicles’ contents as exosomes into the extracellular environment, and thus were responsible for antigen processing in intestinal epithelial cells (3, 29). This could happen as a result of intestinal barrier defects in IBS-D by exposure to harmful intraluminal entities. Scientific and clinical interest in extracellular vesicles has increased rapidly as evidence mounts that they are known to play key roles in the control of coordinating processes: in coagulation, by transferring tissue factor to initiate the extrinsic coagulation cascade (25); in tumor progression, by facilitating the spreading and release of cancer cells to generate metastases (26); and in intestinal epithelial cells, by acting as sensors of antigenic information (19). However, the role of extracellular vesicles in the modulation of intestinal barrier function remains unknown in IBS-D. We hypothesize that it may carry

preassembled complex biological information that elicits healing responses in the neighboring cells. Understanding the interactions between these extracellular vesicles and the intestinal barrier may help characterize the pathogenesis of IBS-D.

Another remarkable finding was that several individual epithelial cells appeared necrotic in the colonic mucosa of IBS-D patients; this phenomenon has not been described previously in the intestinal mucosa of patients with IBS-D. It is interesting to note the similarity of our findings with those previously described in patients with Crohn's disease (CD) (6). Dourmashkin et al. described finding patchy necrosis in the gut of CD patients for the first time, suggesting that the epithelial lesion might be an early step in the pathogenesis of the disease. As we all know, the balance between intestinal epithelial cell proliferation and death is essential for epithelial turnover and barrier homeostasis in the intestinal epithelium (9, 24). The increased presence of necrosis observed by TEM could be indicative that it could cause transient injury to develop in the epithelial barrier and even contribute to corresponding focal permeability defects in patients with IBS-D. Although necrotic cell death in some of the sample fragments was prominent (intestinal mucosa was obtained by biopsy and represents tiny fragments of tissue), most of the cells in the colonic mucosa remained normal. The limitation of TEM should be considered in the study. Abnormalities on the epithelial surface analyzed using TEM may represent focal alterations.

This study has limitations. First, due to methodological limitations, biopsies from IBS-D patients were limited to the rectosigmoid colon; biopsies from other parts of the colon were not obtained. Second, in keeping with the Rome III criteria, the symptom questionnaire did not discriminate between pain and discomfort. Furthermore, patients fulfilling the post-infectious IBS diagnostic criteria were excluded to avoid the probable bias related to colonic mucosa barrier defects.

Taken together, our observations demonstrated intestinal permeability disorders in patients suffering from IBS-D. Our findings first indicated that certain ultrastructural changes were present in the intestinal epithelial cells, potentially representing a pathophysiological mechanism in IBS-D. Delineation of the ultrastructural changes in intestinal barrier function may be critical to understand how development of intestinal barrier dysfunction predisposes to IBS-D and therefore is important for the development of therapeutic strategies.

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Conflict of interest

The authors declare no conflict of interest.

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