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ORIGINAL ARTICLE

Subjects with Diarrhea-Predominant IBS Have Increased Rectal Permeability Responsive to Tryptase

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Abstract

Background and Aims Patients with diarrhea-predominant irritable bowel syndrome (IBS-D) appear to have increased intestinal permeability; it has been suggested that activation of protease-activated receptor-2 (PAR-2) receptors is responsible for this alteration. The aims of this study are to evaluate (1) if rectal (large bowel) permeability is increased in IBS-D and (2) if tryptase plays a critical role in the altered permeability.

Methods Rectal biopsies from 20 patients with IBS-D and 30 subjects without the condition (normal controls) were assessed for macromolecular permeability using horseradish peroxidase in Ussing chambers in the basal state and after addition of drugs to the basolateral side. Reverse-transcription polymerase chain reaction (RT-PCR) was performed using colonic biopsy tissues from patients with IBS-D and normal subjects.

Results When tryptase was added to the basolateral (not mucosal) side of normal rectal biopsy tissues, permeability appeared to be proportional to the increase in tryptase concentration ($P < 0.05$) and was abolished by the addition of tryptase inhibitor (100 μM nafamostat; 1.568 ± 0.874 ng/2 h/mm^2 to 0.766 ± 0.661 ng/2 h/mm^2, $n = 14$, respectively, $P < 0.01$). Intestinal permeability in patients with IBS-D was significantly increased compared with controls ($0.848 \pm 0.0.600$ ng/2 h/mm^2, $n = 21$, $P < 0.01$). Nafamostat significantly reduced the enhanced permeability in IBS-D (0.934 ± 0.589 ng/2 h/mm^2 to 0.247 ± 0.263 ng/2 h/mm^2, $n = 14$, respectively, $P < 0.05$). Transcription levels of *PAR2* measured by RT-PCR did not differ between IBS-D and normal subjects.

Conclusion Tryptase seems to play an important role in the control of human colonic mucosal permeability, and enhanced tryptase activity was responsible for the increased permeability of rectal mucosa in IBS patients.

Keywords Irritable bowel syndrome · Tryptase · PAR-2

Introduction

Irritable bowel syndrome (IBS) is a very common disease, characterized by abdominal pain and altered bowel habits, but the mechanism of the disease is still not clear.

Recent studies have revealed that intestinal permeability is frequently abnormal in patients with diarrhea-predominant IBS [1, 2], and serine protease is thought to play an important role in impaired gut permeability. Fecal supernatants from IBS-D patients are able to induce increased colonic paracellular permeability in colonic strips of mice, and this effect is inhibited by serine protease inhibitors [3]. Furthermore, mucosal content of tryptase was increased in IBS [4], and tryptase from mast cells has been found to control the paracellular permeability of intestine in animal studies [5]. Thus, intraluminal and mucosal proteases are candidate mediators for altered permeability in IBS.

Tryptase is a neutral serine protease and is the most abundant mediator stored in mast cell granules [6]. Mucosal mast cells are also significantly elevated in the

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terminal ileum and cecum of most patients with IBS-D [7, 8], and the close proximity of the mucosal mast cells to the enteric nerves suggests their potential involvement in the development of heightened visceral sensitivity [4]. Therefore, we hypothesized that the tryptase of mast cells can control paracellular permeability of human intestine and that elevated tryptase activity of IBS patients, proportional to the increased numbers of mast cells, can evoke colonic epithelial barrier dysfunction.

The goals of this study are to evaluate (1) if mast cell tryptase can change mucosal permeability in the human colon, (2) if rectal (large bowel) permeability is increased in IBS-D, and (3) if tryptase plays a critical role in the altered permeability in IBS.

Methods

Subjects

Biopsy specimens of the rectum were obtained during routine colonoscopies of 20 patients with IBS-D and 30 control patients without IBS-D who had visited the Kangbuk Samsung Hospital. The IBS patients exhibited symptoms that fulfilled the Rome II criteria. None of the patients had clear histories of abdominal surgery, inflammatory bowel disease, or postinfectious IBS, which is usually diagnosed when patients exhibit at least two of the following: fever, vomiting, diarrhea or positive stool culture at the onset of IBS symptoms, and when the patient's bowel habits have previously been within normal limits. Control subjects had (1) macroscopically and histologically normal colonic mucosa with the exception of incidental hemorrhoids, diverticula, and polyps; (2) no persistent bowel symptoms; (3) no organic or functional bowel disease; and (4) no history of chronic medical disease. Informed written consent was obtained from all patients, and the hospital's local ethics committee approved this study. Biopsy forceps with an opening diameter of 6 mm (FB-25K-1, Olympus, Japan) were used for the procedures. In most cases, three biopsies were taken from rectal segment of IBS-D patients (two for permeability measurement and one for RT-PCR tests).

Permeability Measurement

The biopsies were mounted in modified 2-ml Ussing chambers. After mounting, each half chamber was filled with 2 ml Krebs buffer (KRB), bathing both the mucosal and serosal sides of the specimen. The KRB solution was continuously oxygenated with O_2/CO_2 (95%/5%). pH was kept at 7.4, and a heat block was used to maintain a temperature of 37°C. After a 30-min equilibration period, the KRB in the mucosal compartment was replaced with KRB containing horseradish peroxidase (HRP) at final concentration of 0.4 mg/ml, and the KRB on the serosal side was replaced with fresh KRB. A 0.3 ml sample was collected and replaced with 0.3 ml KRB on the serosal side every 30 min, for a total of 120 min. Samples from the serosal chamber were analyzed enzymatically using a modified Worthington method with *o*-dianisidine dihydrochloride (OPD; Sigma Chemical Co., St Louis, MO, USA) as the substrate. Samples of 50 μl were transferred to microtitre plates. To each well, 100 μl OPD Working Solution (Stable Peroxide Buffer diluted 1:10 in OPD solution) was added and the plate was incubated in a shaker at 300 rpm at room temperature. After 30 min, 100 μl 2.5 M sulfuric acid was added, and 10 min later the absorbance of the colored reaction product was measured at wavelength of 490 nm using a microplate reader (model 680; Bio-Rad Laboratories, Inc.). Blanks were included in each analysis, and all samples were run in duplicate and measured against a standard curve. HRP flux is presented as ng/2 h/mm^2 during steady-state permeation in the 30-min interval.

Tryptase Activity

Aliquots (10 μl) of supernatants of tissue extract were added to 200 μl buffer (50 mmol/l Tris–HCl, pH 7.6, 120 mmol/l NaCl, 20 g/l heparin) containing 0.5 mmol/l mast cell tryptase substrate (tosyl-glycine-proline-arginine-p-nitroanilide) and incubated at room temperature for 17 h. Substrate cleavage was measured using a microplate reader at absorbance of 415 nm, normalized to the protein concentration of the supernatant. Concentrations were calculated automatically in reference to a standard curve. A specific mast cell tryptase inhibitor (nafamostat mesilate) was used to confirm assay specificity.

RNA Isolation and RT-PCR (mRNA Expression Analysis)

Total RNAs from superficial biopsies of rectal mucosa were prepared using Trizol (Invitrogen Life Technologies) according to the manufacturer's guidelines. Complementary DNA (cDNA) synthesis and subsequent PCR were performed using 100 ng total RNA. The expected 240- and 119-bp fragments of *β-actin* and *PAR2* were amplified by PCR using the following primer sets: sense 5′-CTCCATC ATGAAGTGTGACG-3′ (*β-actin*) and antisense 5′-TGCT TGCTGATCCACATCTG-3′ (*β-actin*); sense 5′-CTGGC CATTGGGGTCTTTCTGTTC-3′ (*PAR2*) and antisense 5′-GGCCCTCTTCCTTTTCTTCTCTGA-3′ (*PAR2*). Amplification conditions consisted of a 5 min 95°C initial denaturation step, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. PCR products were visualized on a 2% agarose gel

loaded with 20 µl of the PCR reaction and a 100-bp marker standard.

Statistics

All data are expressed as mean ± standard error of the mean (SEM). Two-tailed Student's *t* tests and χ^2 tests were used for comparison of statistical differences in permeability and tryptase activity between normal subjects and patients with D-IBS, and the paired *t* test was used to determine the significance of a difference in permeability before and after administration of drug. A P value of <0.05 was considered to be significant.

Results

Permeability Change of Normal Rectal Biopsy Tissues According to Tryptase Concentration

We evaluated the permeability of rectal mucosa in control subjects as a function of tryptase concentration. The basal permeability of rectal mucosa was 0.109 ± 0.091 ng/2 h/mm^2 ($n = 14$). When 20 and 50 mU tryptase were added to the basolateral (not mucosal) side of normal biopsy tissues, permeability increased in proportion to tryptase concentration (1.568 ± 0.874 and 2.622 ± 0.535 ng/2 h/mm^2, $n = 14$ and 6, respectively, $P < 0.05$, Fig. 1).

Inhibition of Tryptase Activity by Tryptase Antagonist

After addition of tryptase (20 mU) to the basolateral side of control biopsy tissues, the tryptase antagonist nafamostat mesilate was added to the same side of the Ussing chamber. The increased permeability caused by tryptase was nearly normalized by the addition of the tryptase inhibitor (1.568 ± 0.874 ng/2 h/mm^2 to 0.766 ± 0.661 ng/2 h/mm^2, $n = 14$ respectively, $P < 0.01$, Fig. 2).

Comparison of Permeability and Changes in Permeability After Addition of Tryptase Antagonist Between Patients with IBS-D and Controls

Permeability of rectal biopsy tissues from IBS patients was significantly increased compared with normal controls (0.109 ± 0.091 ng/2 h/mm^2 versus 0.848 ± 0.600 ng/2 h/mm^2, $n = 14$ and 21, respectively, $P < 0.01$, Fig. 3a). The tryptase antagonist nafamostat mesilate nearly abolished the elevated mucosal permeability in D-IBS (0.934 ± 0.589 ng/2 h/mm^2 to 0.247 ± 0.263 ng/2 h/mm^2, $n = 14$, respectively, $P < 0.05$, Fig. 3b) but not in controls (0.068 ± 0.125 ng/2 h/mm^2 versus 0.125 ± 0.145 ng/2 h/mm^2, $n = 8$, respectively, $P > 0.05$, Fig. 3c).

Comparison of Tryptase Activity Between Normal Subjects and IBS Patients

Tryptase activity of biopsy tissues from IBS patients was significantly increased compared with normal controls (4.27 ± 2.12 and 7.24 ± 4.07 mU/mg protein, $n = 18$ and 10, respectively, $P = 0.015$, Fig. 4).

RT-PCR Analysis of *PAR2* mRNA Expression in Human Colonic Mucosa from Normal Subjects and Patients with IBS-D

We quantified messenger RNA (mRNA) expression of *PAR2* from rectal mucosa of control patients and patients with D-IBS using a luminous V3 system normalized to the housekeeping gene *β-actin* (Fig. 5a). There was no significant difference in mRNA expression of *PAR2* between the two groups ($n = 20$, respectively, $P > 0.05$, Fig. 5b).

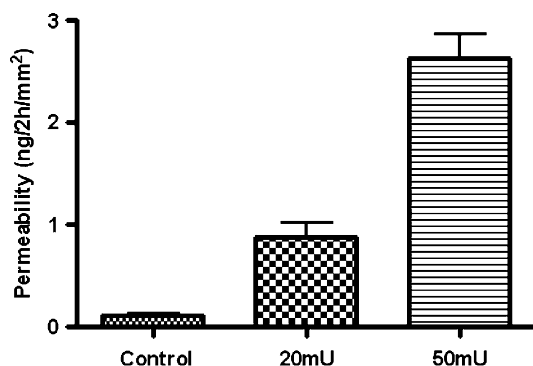

Fig. 1 The basal permeability of rectal mucosa was 0.109 ± 0.091 ng/2 h/mm^2 ($n = 14$). When 20 and 50 mU tryptase were added to basolateral (not mucosal) side of normal biopsy tissues, permeability appeared to be proportional to tryptase concentration (1.568 ± 0.874 and 2.622 ± 0.535 ng/2 h/mm^2, $n = 14$ and 6, respectively, $P < 0.05$)

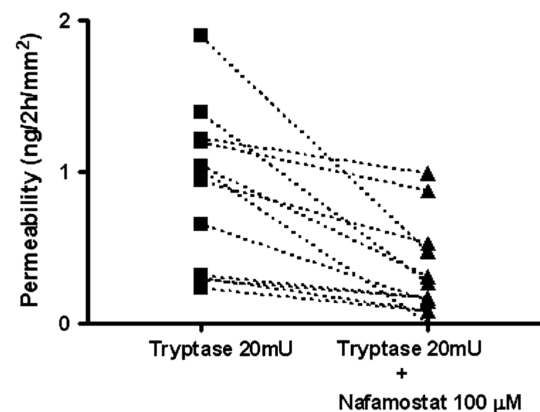

Fig. 2 The tryptase inhibitor nafamostat mesilate (100 µM) nearly abolished the increased permeability of control biopsy tissues that occurred after addition of 20 mU tryptase (1.568 ± 0.874 ng/2 h/mm^2 to 0.766 ± 0.661 ng/2 h/mm^2, $n = 14$, respectively, $P < 0.01$)

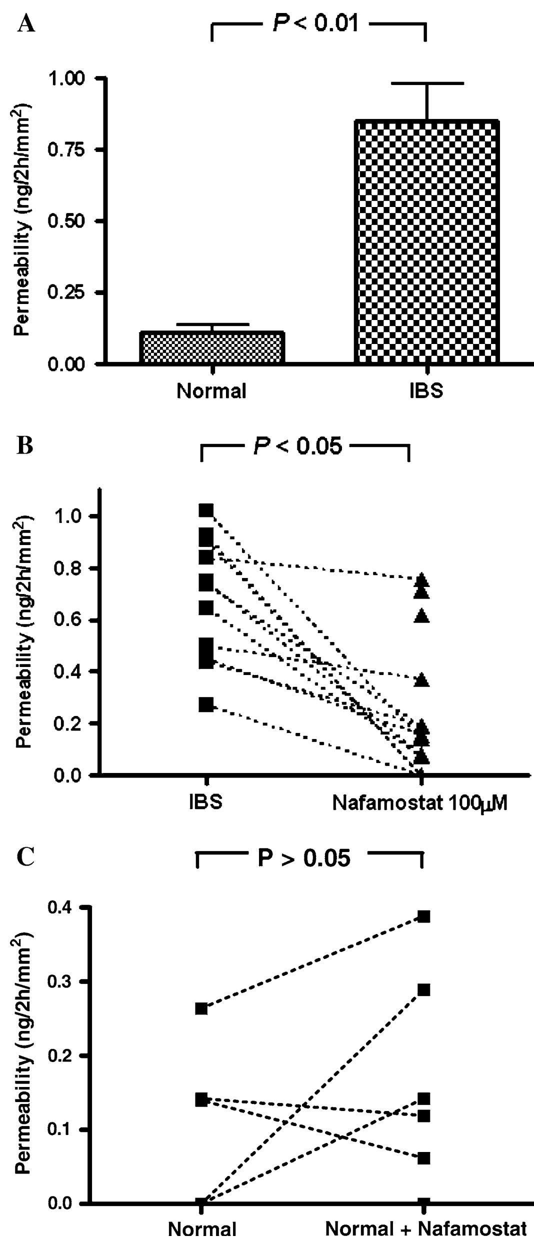

Fig. 3 **a** Permeability was significantly increased in patients with IBS-D compared with controls (0.109 ± 0.091 ng/2 h/mm^2 versus 0.848 ± 0.600 ng/2 h/mm^2, $n = 14$ and 21, respectively, $P < 0.01$) and **b** the tryptase inhibitor nafamostat mesilate significantly reduced the enhanced permeability of IBS-D (0.934 ± 0.589 ng/2 h/mm^2 to 0.247 ± 0.263 ng/2 h/mm^2, $n = 14$, respectively, $P < 0.05$) but not in controls (0.068 ± 0.125 ng/2 h/mm^2 versus 0.125 ± 0.145 ng/2 h/mm^2, $n = 8$, respectively, $P > 0.05$, **c**)

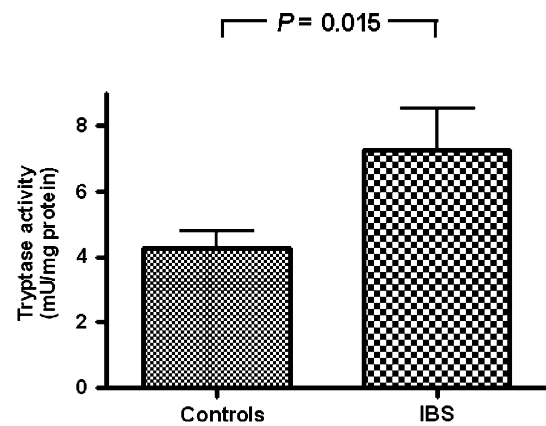

Fig. 4 Tryptase activity of biopsy tissues from IBS patients was significantly increased compared with normal controls (4.27 ± 2.12 and 7.24 ± 4.07 mU/mg protein, $n = 18$ and 10, respectively, $P = 0.015$)

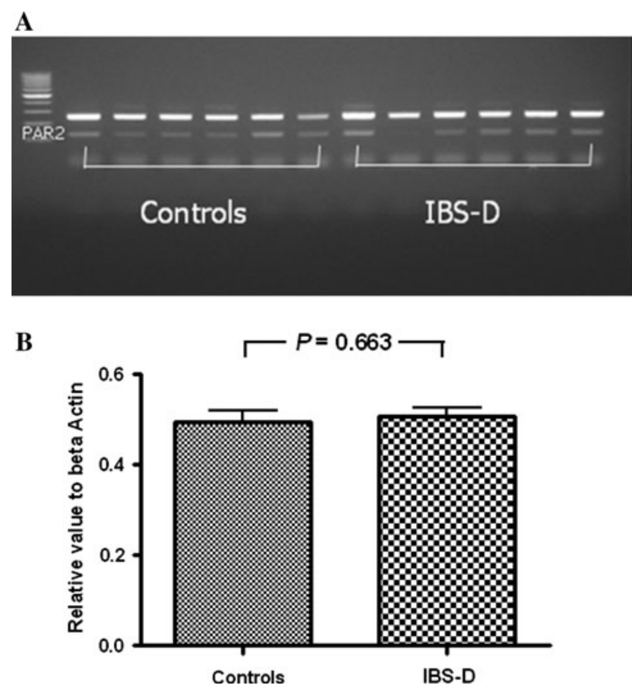

Fig. 5 **a** Quantification of *PAR2* mRNA normalized to the housekeeping gene *β-actin*. **b** RT-PCR analysis showed no difference in the expression of *PAR2* mRNA between the normal controls and patients with IBS-D ($n = 20$, respectively, $P > 0.05$)

Discussion

In this study, we showed that tryptase plays an important role in the control of human colonic mucosal permeability. The elevated permeability in patients with D-IBS is probably due to enhanced tryptase activity, rather than activation of PAR-2, since the enhanced permeability was normalized after administration of a tryptase antagonist, and mRNA expression of *PAR2* in IBS patients was not different from in normal controls.

The standard method currently used to measure intestinal permeability is urinary detection of orally administrated, nonmetabolizable sugar or radioactively labeled substrates such as 51Cr-ethylenediamine tetraacetic acid (EDTA) [9]. However, this method generally lacks the

sensitivity and precision to assess permeability in a specific part of the bowel [10]. In the present study, an Ussing chamber was used to investigate short segment intestinal permeability [11, 12], which has the advantage of providing a controlled environment, without the limitations of an in vivo approach, for studies of mechanisms involved in human intestinal permeability. Selected HRP has frequently been used as a macromolecular marker, because it has similar size to typical food antigens [5, 13–15]. In a previous study, we developed a miniaturized container insert for the Ussing chamber for performing biopsies of human colon tissue. The exposed tissue area was 0.65 mm^2, and findings were reproducible and consistent with previous studies [16].

Our study demonstrates that the increase of permeability of human mucosa is proportional to tryptase concentration and that the increase of permeability is reduced by addition of the tryptase inhibitor nafamostat mesilate. Although the exact mechanism by which mast cells mediate increased mucosal permeability is unknown, it is likely that tryptase plays an important role. Previous studies reported that tryptase is a major protease of human mast cells and that mast cell tryptase increases paracellular permeability [5]. Recent studies also showed that stress and inflammation can induce enhanced permeability to macromolecules by mast cell tryptase [17] and that the protease plays a critical role in disrupting the intestinal barrier and promoting ingress of macromolecules and bacteria that might cause inflammation and infection, among other symptoms [18, 19].

Increasing evidence from recent studies suggests that some forms of IBS are associated with low-grade inflammation of the intestine [20, 21]. Diarrhea-predominant IBS (D-IBS) without an infectious onset was associated with an increase in lamina propria mast cells and T lymphocytes [2, 22]. Additionally, it has been shown that the number of mast cell in rectal mucosa of IBS-D patients is significantly increased compared with in normal controls and that mast cell counts correlate with visceral hypersensitivity [8]. These results are consistent with our findings showing that tryptase activity in rectal mucosa of D-IBS patients is significantly increased compared with normal controls and that enhanced permeability of D-IBS patients is diminished by addition of a tryptase inhibitor.

In the present study, tryptase activity of biopsy tissues from IBS patients was significantly increased compared with normal controls. This result agrees with previous studies indicating increase in protease activity from mucosal biopsies in IBS [4, 23] and further supports the hypothesis that increased tryptase activity from mast cells may be a potent stimulus for increased permeability in IBS. However, luminal bacteria of the intestinal microflora could be one potential source of increased protease [24], and because tryptase inhibitor could not completely abolish the higher permeability in D-IBS, this increase in permeability cannot be a direct effect of tryptase alone. Other factors such as histamine, serotonin, and prostaglandins may also contribute to the increased permeability seen in IBS patients [5], and elevated colonic luminal serine protease activity of IBS-D patients could evoke a PAR-2-mediated colonic epithelial dysfunction as well [3]. This result could partially explain why dexamethasone treatment does not prevent protease-activated receptor-2 (PAR-2) agonist-induced increase in colonic permeability [25].

Activation of PAR-2 receptors on enterocytes induces rearrangements in the cytoskeletal organization of tight junctions and allows the passage of macromolecules across the epithelial barrier [26, 27]. Intracolonic infusion of subinflammatory doses of PAR-2 agonists produced a delayed rectal hyperalgesia involving changes in intestinal permeability [28] and also, in the lung, genetic overexpression of *PAR2* could increase airway hyperresponsiveness, infiltration of eosinophils and mononuclear cells, and levels of proinflammatory cytokines [29]. Therefore, we aimed to determine whether the increased mucosal permeability in IBS patients is associated with overexpression of PAR-2 receptors in the colon using RT-PCR analysis to measure *PAR2* mRNA expression levels. However, we were not able to find any differences in expression levels between the rectal mucosa of normal subjects and that of patients with IBS-D. Thus, we could conclude that the increased permeability of rectal mucosa in IBS was caused not by overexpression of PAR-2 receptors but by enhanced tryptase activity.

An important finding from this study is that the serine protease inhibitor nafamostat mesilate could nearly abolish the increase in mucosal permeability in IBS patients. However, another protease inhibitor, APC366, was found to be ineffective for controlling mucosal permeability. Clinically, nafamostat mesilate is used in the treatment of acute pancreatitis and disseminated intravascular coagulation. At a low dose, nafamostat mesilate strongly inhibits the activity of human tryptase [30] and the permeability of cultured endothelial cells induced by tryptase [31]. Protease activity is responsible for visceral hypersensitivity through the activation of PAR-2 receptors [23]. Increased protease activity also leads to the increase in intestinal barrier permeability that is associated with the symptoms of IBS patients [32]. In consideration of the above results, nafamostat mesilate might be applicable for the treatment of IBS. However, as we discussed earlier, nafamostat mesilate could not totally suppress the increased mucosal permeability of IBS patients, as other mediators including histamine [33] can also have an effect on intestinal mucosal permeability. Further evaluation is needed to elucidate the exact mechanism of increased permeability in IBS.

In vitro measurements of intestinal permeability with biopsy tissues have limitations, including lack of circulation and nervous control. In addition, it is not certain whether the results are representative of the whole segmental permeability in the colon. However, an earlier study verified that human intestinal biopsies have good viability in Ussing chambers, and the chambers can be used to study paracellular permeability to marker molecules [34]. Furthermore, our previous study demonstrated that mast cells in biopsy tissues were significantly increased in the terminal ileum, ascending colon, and rectum of patients with IBS-D, compared with controls, and the increase in the number of mast cells in one part of the colon was significantly related to that in other parts of the colon [8]. However, a few studies reported that the number of mast cells increased a lot in IBS patients only if the right colon was considered [7, 35]. Therefore, if mast cells and their mediators play important roles in the altered permeability in IBS, mucosal permeability of biopsy tissues could be proportional to the number of activated mast cells or the activity of the released tryptase, and altered rectal permeability could be associated with other parts of the colon. A further study to verify the relationship between mast cell counts and tryptase activity in different parts of the colon is underway.

In conclusion, we demonstrated that tryptase is responsible for increased rectal permeability in IBS and that the elevated permeability could be repressed by addition of tryptase inhibitor. Further research is needed to clarify the role of other mediators of mast cells in intestinal permeability and to assess the effectiveness of nafamostat as a treatment option for IBS.

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