THE ROLE OF GUT PERMEABILITY AND SERINE-PROTEASES IN THE PATHOMECHANISM OF IRRITABLE BOWEL SYNDROME AND INFLAMMATORY BOWEL DISEASES

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LIST OF FULL PAPERS THE THESIS IS BASED ON

I. Gecse K, Roka R, Ferrier L, Leveque M, Eutamene H, Cartier C, Ait-Belgnaoui A, Rosztoczy A, Izbeki F, Fioramonti J, Wittmann T, Bueno L. Increased fecal serine-protease activity in diarrheic IBS patients: a colonic luminal factor impairing colonic permeability and sensitivity.
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IF: 9.766

II. M. Dabek, L. Ferrier, R. Róka, K. Gecse, A. Annaházi, J. Moreau, J. Escourrou, C. Cartier, G. Chaumaz, M. Leveque, A. Ait-Belgnaoui, T. Wittmann, V. Theodoru, L. Bueno. Luminal cathepsin G and protease-activated receptor 4: a duet involved in alterations of colonic epithelial barrier in ulcerative colitis.
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IV. Annaházi A, Gecse K, Dabek M, Ait-Belgnaoui A, Rosztóczy A, Róka R, Molnár T, Theodorou V, Wittmann T, Bueno L, Eutamene H. Fecal proteases

from diarrheic-IBS and ulcerative colitis patients exert opposite effect on visceral sensitivity in mice.
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Therapy 2009; 6: 603-613.
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European Gastroenterology & Hepatology Review. 2011;7:26-30.
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INTRODUCTION

The intestinal epithelium is faced with the dual task of providing a barrier while also allowing nutrient and water absorption, therefore its integrity is crucial to maintain physiological function and prevent diseases. Defective epithelial barrier function, which can be measured as increased gut permeability, has been implicated in the pathogenesis of both irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD).

Irritable bowel syndrome is a gastrointestinal disorder characterized by abdominal pain and altered bowel habit, for which there is no apparent structural basis. The diagnosis of irritable bowel syndrome is symptom-based according to the Rome criteria, since 2006 Rome III being the most recent. “Red flag” symptoms for organic disorders are ruled out by careful history taking and thorough physical examination. Rome III defines IBS as recurrent abdominal pain or discomfort lasting for at least 3 days per month in the last 3 months, which is associated with 2 or more of the following characteristics: improvement with defecation, onset associated with change in stool frequency or onset associated with change in stool form. Irritable bowel syndrome affects 5–20% of the population worldwide, thus increasing interest has recently been shown towards its poorly understood pathophysiology and to possible therapeutic approaches of the disease. Due to intensive research of recent decades, there has been a paradigm shift in IBS pathophysiology from considering it a purely psychosocial disturbance to finding organic backgrounds of the disease. By now, genetic factors, altered “brain-gut axis” - both in terms of altered sensory afferent function and central processing resulting in visceral hypersensitivity - abnormal serotonerg neurotransmission, altered gut motility, changes in the gut microflora, stress, intestinal hyperpermeability and mucosal immune activation have all been implicated in

the pathogenesis of IBS. In a preliminary pilot study, it has been shown that fecal supernatants of diarrhea-predominant IBS (IBS-D) patients have a substantially higher serine protease activity, similar to that of patients with ulcerative colitis (UC), when compared with healthy subjects or other subgroups of IBS patients. Certain serine proteases are signaling molecules that cleave protease-activated receptors (PARs), a family of G-protein coupled receptors with a widespread distribution. Four members of the PAR family have been identified so far in human tissues, of which PAR-2 and 4 are highly expressed on intestinal epithelial cells. The primary activating protease of PAR-2 is trypsin, however, serine proteases of other endogenous and/or bacterial origin are also able to cleave the receptor. Activation of PAR-2 modulates several gastrointestinal functions, such as motility, ionic exchange, paracellular permeability, sensory functions and inflammation. Intracolonic but not intraperitoneal administration of the synthetic selective PAR-2 agonist, SLIGRL (H-serine-leucine-isoleucine-glycine-arginine-leucine-OH), produces delayed visceral hyperalgesia in rats associated with increased paracellular permeability. In the intact epithelium the paracellular space between adjacent cells is sealed by dynamically changing tight junctions at the luminal aspect of the apical junction complex, which forms a selectively permeable barrier and is structurally related to the perijunctional actomyosin ring. The integrity of the epithelial barrier is dependent upon the contraction of this perijunctional actomyosin ring and subsequent physical tension on the tight junction, an event that involves the phosphorylation of myosin light chain (MLC), initiated by MLC kinase (MLCK).

Interestingly, both visceral hypersensitivity and impaired intestinal permeability are commonly observed features of IBS. Gut permeability has been reported to be enhanced in 50% of post-infectious IBS patients, in agreement with the study showing increased small intestinal permeability in

both the post-infectious and sporadic forms of IBS, characteristically in the diarrhea-predominant subtype, the same subpopulation where increased serine protease activity has been detected. In accordance, the report on the "Walkerton epidemic" - a waterborne outbreak of acute gastroenteritis in Walkerton, Ontario - proved subtle increase in small intestinal permeability in a large number of patients with IBS, however in vitro studies suggest enhanced permeability in colonic biopsies of IBS patients compared to healthy subjects. Therefore it seems that gut permeability in IBS is altered, though the data on the subgroup of IBS patients affected and the exact localization of the defective barrier are still contradictory. It is also well established that impaired intestinal barrier function could facilitate the passage of luminal antigens and lead to mucosal immune response. Furthermore, there is growing evidence for microinflammation of the intestinal and colonic mucosa to play a role in IBS pathogenesis. Thus identifying the role of defective mucosal barrier in IBS pathomechanism and symptom generation may be an important landmark in better understanding of the disease.

Inflammatory bowel disease is a chronic inflammatory condition of the intestines that is characterized by remission and relapses and distills clinically into one of the two major subtypes of disease: ulcerative colitis and Crohn‘s disease (CD). Current understanding of the underlying pathomechanisms in IBD is that dysregulated mucosal immune response leads to barrier defect triggered by antigenic components of the normal commensal microbiota that reside within the intestine in a genetically susceptible host. Epithelial barrier impairment is considered important in IBD as it leads to increased luminal antigen exposition of the lamina propria, i.e. immune cells, including large number of neutrophils, which further aggravate both the inflammatory process and the hyperpermeability. Neutrophils are characterized by two major granule populations, primary (azurophil) and secondary (specific) granules, formed at

different stages of neutrophil maturation. Cathepsin-G (Cat-G), a serine protease, makes up approximately 20% of the neutrophil azurophil granule proteins and it also plays an important role in neutrophil function during inflammatory processes, including degradation of extracellular matrix components and cytokines, modulation of integrin clustering on neutrophils and direct chemoattraction of T cells and other leukocytes. Besides trypsin and thrombin, proteinase-activated receptor 4 is also activated by the neutrophil derived serine protease cathepsin-G.

There is strong evidence for barrier dysfunction in IBD: hyperpermeability in non-involved segments of the intestine of CD patients as well as in first degree relatives has been reported and increased permeability have also been associated with an increased risk of relapse. Data are however less abundant on paracellular permeability regarding UC. Increase in gut permeability has previously been reported in clinically active UC, which was also shown to correlate with disease severity. Still, gut permeability has not yet been evaluated in remission of the disease.

AIMS

The studies were conducted (i) to investigate the origin of elevated fecal serine protease activity in IBS-D patients, (ii) to evaluate if this elevated colonic luminal serine protease activity may be sufficient to trigger alterations in colonic permeability and sensitivity in mice, (iii) to examine the possible involvement of PAR-2 activation in this process and (iv) to analyze the underlying molecular mechanisms in the tight junction. We also aimed to (v) evaluate whether the high colonic luminal serine-protease activity in UC may contribute to increased gut permeability and (vi) to examine the possible

involvement of cathepsin-G and PAR-4 in this process. Furthermore, we aimed (vii) to measure intestinal and colonic permeability of patients with IBS of the diarrhea and of the constipation-predominant subtype in vivo, (viii) to investigate possible correlation between increased gut permeability and clinical symptoms in IBS-D patients and (ix) to measure gut permeability in patients with ulcerative colitis in remission.

PATIENTS AND METHODS

1. Patient selection

1.1. Fecal serine proteases in IBS and in UC, their role in mediating increased colonic permeability and sensitivity in murine model

Demographic data of the patients enrolled in the study are summarized in Table 1. Patients fulfilling the Rome II criteria for IBS (patient screening was carried out in 2005-2006) participated in the study. All participants provided medical history and underwent physical examination. In IBS patients other gastrointestinal disorders were excluded by detailed blood and stool analyses, serological assays for coeliac disease, lactose–hydrogen breath test and colonoscopy. Active UC was assessed clinically and endoscopically. The study protocol was approved by the Ethical Committee of the University of Szeged. All subjects provided written and informed consent to participate.

Group	Number of patients	Age mean (range)	Sex ratio (M/F)
IBS-D	24	49 (19-75)	9/42
IBS-C	18		
IBS-A	10		
UC	17	41 (18-79)	6/11
INF	23	52 (19-90)	12/11
Healthy subjects	25	44 (30-65)	4/21

Table 1. Demographic data. IBS-D: diarrhea-predominant IBS patients, IBS-C: constipation-predominant IBS patients, IBS-A: IBS patients with altered bowel habit, UC: patients with ulcerative colitis, INF: patients with acute infectious diarrhea.

1.2. In vivo gut permeability in IBS and in inactive ulcerative colitis

Demographic data of the patients enrolled in the study are summarized in Table 2. Thirty patients fulfilling the Rome III criteria for IBS participated in the study, none of which related the onset of their symptoms to infectious gastroenteritis. According to the Rome III criteria introduced in 2006, symptoms are expected to originate 6 months before diagnosis, which is a less restrictive timeframe compared to the Rome II criteria (12 weeks of symptoms over 12 months). In addition, according to Rome III criteria IBS subtyping should be based on stool consistency, which results in four subgroups, namely diarrhoea predominant (IBS-D), constipation predominant (IBS-C), subjects with mixed pattern (IBS-M) and unsubtyped IBS (IBS-U). Still, bowel subtyping used in Rome II for IBS-D and IBS-C remained acceptable. Other gastrointestinal disorders were excluded by detailed blood and stool analyses, serological assays for celiac disease, lactose-hydrogen breath test and colonoscopy. Patients with inactive ulcerative colitis (partial Mayo score ± SEM: 1.3±0.2; CRP (mg/dl) ± SEM: 3.8±1.3) were previously shown to have

either left-sided colitis or pancolitis. Voluntary subjects, free of any gastrointestinal symptoms, served as controls. Patients and voluntary subjects with impaired renal function, alcohol consumption, using NSAIDs, prokinetics, antihistamines or immunosuppressive agents were excluded from the study. UC patients were required to be exclusively on 5-ASA maintenance therapy. The study protocol was approved by the Human Investigation Review Board, University of Szeged. All subjects provided written and informed consent to participate.

Group	Number of patients	Age mean (range)	Sex ratio (M/F)
IBS-D	18	49 (25-68)	6/12
IBS-C	12	56 (37-65)	2/10
UC	13	47 (29-72)	3/10
Healthy subjects	10	49 (38-65)	2/8

Table 2. Demographic data

2. Methods

2.1. Fecal samples

Only feces collected in situ, or collected at home and transported within 1 h after defecation to the First Department of Internal Medicine in Szeged, Hungary, were used. Samples were stored at -80°C until transportation on dry ice to INRA Toulouse, France. Upon arrival, 1g of fecal sample was thawed, dissolved, and homogenised in 7ml of Tris buffer, centrifuged (4500 rpm, 10 min, +4C) and filtered (0.2 μm, Nalgene, (Adventure 16, San Diego, California, USA)). The acquired supernatants were used for measuring total protease activity, serine protease activity, polymorphonuclear neutrophil (PMN) and pancreatic elastase, myeloperoxidase (MPO), calprotectin and human secretory leucocyte protease inhibitor (SLPI) activity.

2.2. Measurement of fecal enzymatic activities

To measure total fecal serine protease activity, supernatants of fecal homogenates (25 ml) were incubated with 1 ml of reaction buffer (0.15 M NaCl and 20 mM Tris-HCl, pH 8.3) and 1ml of 0.5% (w/v) azocasein (Sigma, St Quentin Fallavier, France) at 40°C. The reaction was stopped after 20 minutes with 1ml of 10% (v/v) trichloracetic acid (TCA, Sigma). Following centrifugation, absorption of the clear supernatant was measured at 366 nm. Enzymatic activities of the supernatants were normalized to protein content. To determine whether protease activity was dependent upon serine proteases, measurements were done after preincubation for 30 min with selective serine protease inhibitors, AEBSF (4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride) and a mixture of soybean trypsin inhibitor (SBTI) and aprotinin (Sigma). Protease activity was expressed as units per milligram of protein, standardized against activity elicited by 1U of standard trypsin. Pancreatic elastase-1, SLPI, PMN elastase and calprotectin concentrations were assayed by ELISA (Schebo-Tech, Giessen, Germany; R& D Systems, Lille, France; Immundiagnostik AG, Bensheim, Germany; HyCult Biotechnology, Uden, The Netherlands, respectively). Fecal MPO activity was measured as described earlier. Cat-G activity was measured in fecal supernatants from patients with UC and healthy subjects, using N-succinyl-Ala-Ala-Pro-Phe p Nitroanilide (Sigma) as a substrate. Enzymatic activity was measured at 410 nm for 5 minutes at 37°C.

2.3. Animals

Congenic 6–9-week old male C57BL/6J wild type (Janvier, Le Genest St-Isle, France) and PAR-2-deficient mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used. The genetic status of the PAR-2-deficient mice was confirmed by PCR. Mice were housed in polypropylene cages in a light- and

temperature-controlled room (12 h/12 h cycles; 20±2°C), were fed standard pellets (Harlan Teklad, Bicester, Oxon, UK), and water was provided ad libitum. The experimental protocols described in the study were approved by the local Institutional Animal Care and Use Committee.

2.4. Visceral sensitivity model

Under xylazine/ketamine anaesthesia (both 1.2 mg, subcutaneously), two nickel-chrome electrodes were implanted into the abdominal external oblique muscle and a third into the abdominal skin, and were exteriorized on the back of the neck. On the fifth to seventh postoperative day, colorectal distensions were used as noxious stimuli to evaluate visceral hyperalgesia by electromyographic (EMG) recording. Under sodium pentobarbital anaesthesia (10mg, intraperitoneally), polyethylene perfusion and distension catheters (Fogarty catheter for arterial embolectomy, 4F, Edwards Lifesciences, Nijmegen, The Netherlands) were inserted into the colon. Animals received 0.3 ml of fecal supernatants of IBS-D or IBS-C (constipation-predominant IBS) patients, of healthy subjects or of IBS-D patients previously incubated with serine protease inhibitors, SBTI and aprotinin. The colorectal distension procedure started 60 min after the infusion of the supernatants had finished with volumes progressively increasing in 0.02 ml steps, from 0 to 0.12 ml, each step lasting 10s with 5 min non-distension periods in between. During the distension periods, the striated muscle's EMG activity was recorded and analyzed according to Larsson *et al*. Basal EMG activity was subtracted from the EMG activity registered during the periods of distension.

2.5. In vitro permeability model

Mice were sacrificed by cervical dislocation and the distal part of the colon was removed. Colonic strips were mounted with a flux area of 0.3 cm2 in

Easymount Ussing-type chambers (Physiologic Instruments, San Diego, California, USA), bathed in Krebs solution and oxygenated at a maintained temperature of 37°C. After allowing 15 min for equilibrium, one-fifth of the initial volume of the buffer solution (1ml) of the apical compartment (mucosal side) was replaced with physiological saline or supernatants (500ul) and fluorescein isothiocyanate (FITC)-labelled dextran (500ul) (4000 Da, 0.022 g/ml, Sigma). 60 minutes later fluorescent intensity was measured on the serosal side of the chamber. Supernatants derived from healthy subjects, from IBS patients or from UC patients. IBS-D supernatants were alternatively pre-incubated with protease-inhibitors soybean trypsin inhibitor (SBTI) and aprotinin for 30 minutes on ice and than added to the mucosal side of the colonic strip. To assess the potential role of PAR-1, PAR-2 and PAR-4 in the effect of UC fecal supernatants on colonic paracellular permeability, selective receptor antagonists were used: PAR1 antagonist FLLRN (Phe-Leu-Leu-Arg-Asn, 10μmol/L, Peptides International, Louisville, KY), PAR2 antagonist FSLLRY (Phe-Ser-Leu-Arg-Tyr, 10μmol/L, Bachem, Weil am Rhein, Germany) and P4-pal10 pepducin (final concentration 1μmol/L) (NeoMPS, Strasbourg, France) were added to the mucosal side of the chamber prior to the administration of fecal supernatants to the mucosal side. The effect of the PAR-4 agonist peptide (Ala-Tyr-Pro-Gly-Lys-Phe-NH2, 50 μmol/L, Sigma) was also assessed, as well as the effect of a selective cathepsin-G inhibitor (SCGI): UC fecal supernatants were pre-incubated for 30 minutes on ice with the inhibitor (0.2mmol/L) and then the mixture was added to the mucosal side of the colonic strip.

2.6. Immunohistochemistry of pMLC and ZO-1

At 1 and 4 h after the intracolonic infusion of supernatants of IBS-D patients or healthy controls, saline, SLIGRL (5 mg) or IBS-D supernatants previously

incubated with serine protease inhibitors, the mice were sacrificed and the distal colon was removed. For both phosphorylated MLC (pMLC) and ZO-1 immunolabelling, samples were fixed in buffered paraformaldehyde (4%), incubated in 30% sucrose (24 h, +4°C), embedded (Tissue Tek medium) and frozen in isopentane at -45°C. Cryostat sections (7um) were fixed with acetone (10 min, -20°C), hydrated in phosphate-buffered saline (PBS) and treated with 4 mg/ml sodium borohydride (45min, +4°C). Sections were permeabilized with PBS–0.5% Triton X-100 and incubated in blocking solution (PBS containing 1% bovine serum albumin). For pMLC staining, samples were then incubated with goat anti-pMLC antibodies (1/100, SantaCruz, Santa Cruz, California, USA) followed by incubation with biotin-conjugated IgG donkey anti-goat antibody (1/1000, Interchim, Montlucon, France). Sections were rinsed in NaHCO3 (0.1M, pH 8.2) and incubated with FITC-conjugated avidin (1/500) diluted in the same solution. For ZO-1 labelling, sections were incubated with rabbit anti-ZO-1 antibodies (1/500, Zymed, San Francisco, California, USA) followed by incubation with Alexa fluor 488-conjugated IgG donkey anti-rabbit antibodies (1/2000, Molecular Probes, Cergy-Pontoise, France). All sections were mounted in Vectashield HardSet Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, California, USA) and examined under a Nikon 90i fluorescent microscope.

2.7.Western blot for pMLC

Colonic mucosa was collected from mice 1h after intracolonic infusion with fecal supernatants of healthy subjects or IBS-D patients, or saline. Proteins were extracted with RIPA buffer and quantified. Following the Laemmli method, equal amounts of protein extracts were electrophoresed by 12% SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and then electrotransferred onto Hybond-P membrane (GE Healthcare, Bordeaux,

France). After saturation, the membrane was incubated with anti-pMLC primary antibody (1/1000, Biosource, Worcester, Massachusetts, USA) and peroxidase-conjugated goat anti-rabbit secondary antibody (1/1000, Millipore, St Quentin en Yvelines, France). The membrane was developed with SuperSignal Reagent (Pierce, Prebieres, France). Integrated density values were assessed by ImageJ 1.37 software (NIH, Bethesda, Maryland, USA).

2.8. In vivo permeability measurement with ^{51}Cr-EDTA

To measure intestinal and colonic permeability after an overnight of fasting participants emptied their bladders and consumed ^{51}Cr-EDTA (Perkin Elmer Life Sciences, Boston, MA, USA) of 1.8 MBq activity dissolved in 100 ml of water, followed by 200 ml of standard meal (Nutridrink, Nutricia, Budapest, Hungary) containing 300 kcal. Study participants were requested to restrain from drinking for 3 hours and from eating for 5 hours. Gut permeability was evaluated by measuring 24-hour urine excretion of orally administered ^{51}Cr-EDTA, where time periods were chosen to relate to permeability within the proximal (0-3h) and distal (3-5h) small intestine and the large bowel (5-24h). Urinary output was recorded for each period and the radioactivity of 1 ml aliquots were counted by a gamma-counter (Packard Cobra, Canberra Packard, UK) in duplicates. Gut permeability was expressed as percentage of urinary excretion of the orally administered dose of ^{51}Cr-EDTA (%).

2.9. Evaluation of symptoms in IBS-D patients

IBS-D patients were asked to fill out a questionnaire, regarding their clinical symptoms at the time of the permeability measurement. Stool frequency (/ week) and consistency (according to the Bristol stool scale), frequency of abdominal pain, distension and bloating (/week), intensity of abdominal pain,

distension and bloating and quality of life (visual analogue scale /VAS/; %) were the symptoms being evaluated and correlated to colonic permeability.

3. Statistical analysis

All data are presented as means ± SEM. For statistical analysis, Prism 4.0 (GraphPad, San Diego, California, USA) was used. Multiple comparisons for fecal enzymatic activities of different patient groups and integrated optical density values of pMLC western blots were analyzed by repeated measures of one-way analysis of variance (ANOVA), followed by Tukey's posttest or Kruskal–Wallis posttest (for MPO activity). Statistical significance for visceral hypersensitivity results was established by using two-way ANOVA, followed by Bonferroni posttest. In vitro permeability results were analyzed with one-way ANOVA, followed by Tukey posttest or two-tailed unpaired t test. Multiple comparisons for in vivo permeability of different patient groups were analyzed by repeated measures of one-way ANOVA, followed by Tukey's posttest. Unpaired t-test was used to evaluate colonic permeability data in subgroups of UC patients. Linear regression was applied to establish correlation between clinical symptoms and permeability. Statistical significance was accepted at $p < 0.05$.

RESULTS

1. Fecal serine protease activity

In healthy subjects the total fecal serine protease activity was 698 U/mg of protein. This serine protease activity was significantly greater in IBS-D patients (2079 U/mg, $p<0.001$) and in UC patients (2193 U/mg, $p<0.01$) compared with healthy controls; a similar increase was not present regarding

the IBS-C, IBS-A subgroups or patients with acute infectious diarrhea (INF). Addition of the serine protease inhibitor AEBSF abolished this increased protease activity in IBS-D and UC supernatants. A similar inhibition was obtained with preincubation with SBTI and aprotinin, two common serine protease inhibitors validated for human use. Regarding the potential origin of the elevated serine protease activity, no significant difference was observed in fecal pancreatic elastase-1 concentration in UC or in any subgroups of IBS patients when compared with controls. However, pancreatic elastase-1 concentration was significantly decreased in INF patients ($p<0.05$), which might be attributed to the diluted fecal content (i.e. profuse watery diarrhea) and restricted diet. PMN-derived elastase had a significantly elevated fecal concentration in UC and INF patients ($p<0.05$), but not in any subgroup of IBS patients. Human fecal SLPI activity showed no significant difference in any of the studied groups of patients when comparing them with control subjects. Fecal inflammatory markers, such as human calprotectin and MPO, showed a significant increase in UC ($p<0.001$ and $p<0.05$, respectively) and INF patients ($p<0.05$ and $p<0.001$, respectively), but we found no increase in any of the IBS subgroups.

2. Visceral hypersensitivity is triggered by elevated serine protease activity of IBS-D supernatants and is dependent on mucosal PAR-2 expression

Intracolonic infusion of fecal supernatants from IBS-D patients administered prior to colorectal distensions in mice significantly increased the abdominal muscle EMG response (a valid criterion representing nociception) at low distension volumes, namely at 0.02, 0.04 and 0.06 ml, compared with the intensity of muscle contractions in animals treated with fecal supernatants of control subjects (0.02 ml, 30.6 (5.9) mV/s vs 3.5 (1.7) mV/s; 0.04 ml, 73.4 (7.9) mV/s vs 18.9 (7.5) mV/s; 0.06 ml, 107.4 (4.4) mV/s vs 53.2 (14.3) mV/s;

$p<0.05$, $p<0.001$ and $p<0.001$, respectively). Conversely, colonic instillation of IBS-C supernatants did not evoke alteration of visceral sensitivity to colorectal distension. Incubation of fecal supernatants from IBS-D patients with serine protease inhibitors prior to colonic infusion, prevented the increased EMG response to low volumes of distension (0.02 ml, 2.8 (1.4)mV/s, 0.04 ml, 49.8 (2.9) mV/s; $p<0.01$, $p<0.05$, respectively), while protease inhibitors per se had no effect (data not shown). Furthermore, IBS-D supernatants failed to induce allodynia or visceral hypersensitivity in PAR-2-deficient mice (0.02 ml, 4.6 (3.5) mV/s; 0.04 ml, 41.4 (7.0) mV/s; 0.06 m, 59.4 (14.2) mV/s).

3. Increase in colonic paracellular permeability (CPP) is evoked by elevated serine protease activity of IBS-D fecal supernatants and is PAR-2 dependent

Administration of fecal supernatants from healthy subjects to the mucosal side of the murine colon mounted in Ussing chambers did not significantly alter CPP compared with saline (128% vs. 100%; ns.). In contrast, addition of fecal supernatants from IBS-D patients significantly increased the FITC–dextran flux compared with the administration of supernatants from healthy subjects or saline (192%, $p<0.05$). Comparatively, supernatants from IBS-C patients failed to induce such an increase in colonic permeability (137%; ns.). Previous incubation of the supernatants from IBS-D patients with SBTI and aprotinin prior to administration significantly decreased the elevated CPP evoked by supernatants from IBS-D patients (111% vs. 202%, $p<0.01$). No increase in CPP was observed in response to fecal supernatants from IBS-D patients on colonic strips of PAR-2-deficient mice in contrast to their wild-type strain (127% vs. 248%, $p<0.05$).

4. Increased rapid phosphorylation of MLC and delayed redistribution of ZO-1 in epithelial cells after mucosal exposure to IBS-D fecal supernatants

One hour after intracolonic infusion with supernatants from IBS-D patients there was a pronounced and diffuse labelling of pMLC in epithelial cells, similar to that observed after the infusion of SLIGRL used as a positive control for PAR-2 activation, and inhibited by preincubation with serine protease inhibitors. Administration of supernatants from healthy subjects or saline failed to evoke an increase in pMLC immunostaining, which remained restricted to the tight junction area.

Four hours after intracolonic infusion of supernatants from healthy subjects or saline, selective immunostaining of the tight junction protein ZO-1 showed labelling that was restricted to the surface of epithelial cells. In contrast, intracolonic infusion of supernatants from IBS-D patients resulted in a marked labelling of the intracellular compartment, similarly to that experienced after the infusion of SLIGRL, suggesting intensive internalisation of the protein. Previous incubation of fecal supernatants from IBS-D patients with serine protease inhibitors prevented the occurrence of a marked intracellular labelling of colonocytes. Western blotting showed an increased level of pMLC in the colonic mucosa of mice 1h after the intracolonic infusion of supernatants from IBS-D patients compared with those infused with supernatants from healthy controls ($p<0.001$). There was no significant difference in the level of pMLC in the colonic mucosa of mice that were infused with supernatants from healthy subjects or IBS-D supernatants previously incubated with a mixture of serine protease inhibitors.

5. Increase in colonic paracellular permeability (CPP) is evoked by cathepsin-G of UC fecal supernatants and is PAR-4 mediated

Administration of fecal supernatants from healthy subjects to the mucosal side of murine colon mounted in Ussing chambers did not significantly alter CPP, as compared with saline (1.58 ± 0.29 vs. 2.00 ± 0.28nmol/h/cm^2, ns). In contrast, addition of fecal supernatants from UC patients significantly increased the FITC-dextran flux compared with supernatants from healthy subjects (3.34 ± 0.47nmol/h/cm^2; $p<0.05$) and saline ($p<0.01$). The addition of PAR-1 antagonist FLLRN (10μmol/L) and PAR-2 antagonist FSLLRY (10μmol/L) did not significantly alter the increased CPP triggered by UC fecal supernatants. In contrast, pepducin P4pal-10 significantly reduced the effect of UC fecal supernatants on permeability (1.84 ± 0.20nmol/h/cm^2; $p<0.05$). Finally, PAR4 activating peptide (AYPGKFNH$_2$; 50 μmol/L) increased the dextran flux across mice colonic epithelium by 199% in comparison with vehicle (2.69 ± 0.17 vs. 0.90 ± 0.17nmol/h/cm^2, $p<0.001$). Fecal supernatant from patients with UC showed high activity, whereas those from healthy subjects presented weak ability to cleave the substrate N-succinyl-Ala-Ala-Pro-Phe p-Nitroanilide, which is widely used to assess Cat-G activity (28.34 ± 3.05 vs. 2.02 ± 0.22, $p<0.001$). Moreover, SCGI decreased enzymatic activity in UC supernatants by 43% (16.14 ± 2.02, $p<0.01$), showing that Cat-G is present in significant amounts within the lumen in UC, but not in healthy subjects. Preincubation of UC fecal supernatants with SCGI abolished the effect of UC fecal supernatants by 77% on paracellular permeability (3.49 ± 0.53 vs. 2.01 ± 0.30, $p<0.05$).

6. In vivo gut permeability in IBS and in inactive UC patients

Twenty-four hours urinary excretion of orally administered ^{51}Cr-EDTA showed significant increase in the IBS-D and UC groups of patients compared

to control subjects (3.93±0.43 and 5.39±0.61 vs. 1.97±0.33%, $p<0.05$ and $p<0.001$ respectively). Gut permeability in IBS-C patients remained as low as those of controls showing no significant difference (1.34±0.2%). Results were consistent with the above when time periods were chosen to relate to permeability within the proximal (0-3h) and distal (3-5h) small intestine and the large bowel (5-24h) during twenty-four-hour urine excretion of orally administered ^{51}Cr-EDTA. There was no significant difference in the proximal small intestinal permeability in IBS-D and inactive UC patients compared to controls (0.63±0.08 and 0.82±0.09 vs. 0.63±0.1%, respectively). However, proximal small intestinal permeability of IBS-C patients was significantly decreased compared to controls (0.26±0.05%; $p<0.05$). Gut permeability did not show any significant difference regarding the distal small intestine in the diarrhea- and constipation-predominant subgroups of IBS patients, and patients with inactive UC compared to control subjects (0.61±0.12, 0.39±0.08, 0.83±0.09 vs. 0.43±0.07%, respectively). Colonic permeability of IBS-C patients remained as low (0.69±0.12%), as those of control subjects, showing no significant difference. On the contrary, colonic permeability of IBS-D patients proved to be significantly higher compared to healthy controls (2.68±0.35 vs. 1.04±0.18%; $p<0.05$). Furthermore, colonic permeability of patients with inactive UC was also found to be significantly elevated compared to control subjects (3.74±0.49 vs. 1.04±0.18%; $p<0.001$). There was no significant difference in colonic permeability between patients with previous endoscopic diagnosis of left-sided colitis or pancolitis (3.26±0.43 vs. 4.31±0.94%, ns.).

7. Correlation between increased gut permeability and clinical symptoms in IBS-D patients

Stool consistency, frequency of abdominal pain, distension and bloating, intensity of abdominal pain, distension and bloating or quality of life did not show correlation with increased colonic permeability in IBS-D patients. Nevertheless, stool frequency showed good correlation with colonic permeability in IBS-D patients ($r=0.62$; $p=0.005$). Colonic permeability of inactive UC patients did not show correlation with stool frequency.

CONCLUSIONS

To summarize the new results, we have shown that (i) serine protease activity can be used as a biomarker to distinguish between IBS-D and acute infectious diarrhea, and permits differentiation of IBS-D and UC patients in the absence of increased fecal inflammatory markers; (ii) elevated luminal serine protease activity seen in IBS-D patients is sufficient to trigger an increase in colonic permeability and subsequent visceral hypersensitivity in mice, suggesting similar effects in humans; (iii) these effects of luminal serine proteases are PAR-2-mediated; (iv) colonic exposure to supernatants from IBS-D patients triggers rapid phosphorylation of MLC and delayed internalisation of ZO-1 in colonocytes in vivo; (v) elevated serine-protease activity seen in UC is able to trigger epithelial barrier disruption; (vi) this barrier disruption is evoked by the neutrophil-derived mediator, cathepsin-G via PAR-4 activation; (vii) impaired epithelial barrier function is localized to the colon and is restricted to the diarrhea-predominant subtype of IBS patients; (viii) increased colonic permeability in IBS-D patients correlates with stool frequency and (ix) colonic epithelial barrier is also compromised in patients with UC in remission.

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