Sodium absorption stimulator prostasin (PRSS8) has an anti-inflammatory effect via downregulation of TLR4 signaling in inflammatory bowel disease

(short title) Role of prostasin in DSS-colitis

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Abstract

(Backgrounds and aims) Prostasin (PRSS8) is a stimulator of epithelial sodium transport. In this study, we evaluated alteration of prostasin expression in the inflamed mucosa of patients with inflammatory bowel disease (IBD) and investigated the role of prostasin in the gut inflammation. **(Materials and methods)** Prostasin expression was evaluated by immunohistochemical staining. Dextran sodium sulfate (DSS)-colitis was induced in mice lacking prostasin specifically in intestinal epithelial cells (*PRSS8*$^{\Delta IEC}$ mice). **(Results)** In colonic mucosa of healthy individuals, prostasin was strongly expressed at the apical surfaces of epithelial cells, and this was markedly decreased in active mucosa of both ulcerative colitis and Crohn's disease. DSS-colitis was exacerbated in *PRSS8*$^{\Delta IEC}$ mice compared to control *PRSS8* $^{lox/lox}$ mice. Toll-like receptor (TLR) 4 expression in colonic epithelial cells was stronger in DSS-treated *PRSS8*$^{\Delta IEC}$ mice than in DSS-treated *PRSS8* $^{lox/lox}$ mice. NF-κB activation in colonic epithelial cells was more pronounced in DSS-treated *PRSS8*$^{\Delta IEC}$ mice than in DSS-treated *PRSS8*$^{lox/lox}$ mice, and the mRNA expression of inflammatory cytokines was significantly higher in DSS-treated *PRSS8*$^{\Delta IEC}$ mice. Broad-spectrum antibiotics treatment completely suppressed the exacerbation of DSS colitis in *PRSS8*$^{\Delta IEC}$ mice. The mRNA expression of tight junction proteins and mucosal permeability assessed using FITC-dextran were comparable between DSS-treated *PRSS8* $^{lox/lox}$ and DSS-treated *PRSS8*$^{\Delta IEC}$ mice. **(Conclusion)** Prostasin has an anti-inflammatory effect via downregulation of TLR4 expression in colonic epithelial cells. Reduced prostasin expression in IBD mucosa is linked to be a deterioration of local anti-inflammatory activity and may contribute to the persistence of mucosal inflammation.

(Key words) prostasin, TLR4, NF-κB, mucosal permeability

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing inflammatory disorders characterized by bloody diarrhea and abdominal pain and cramping (1-3). The exact cause of IBD remains unclear, but its etiology is closely associated with an excess immune response in the mucosa against dietary factors and gut microbes in persons with a genetic predisposition (1-3).

Prostasin [protease serine S1 family member 8 (PRSS8)] is an extracellular serine protease, expressed mainly in mammalian epithelial cells of the prostate, kidneys, lungs, and distal colon as a 40kDa glycosylphosphatidylinositol (GPI)-anchored protein(4). Prostasin is a channel activating protease which stimulates the amilorides-sensitive epithelial sodium channels (ENaC) and provokes electrogenic sodium transport (5, 6). ENaC consists of α, β and γ homologous subunits (7) and prostasin plays a crucial role in full activation of ENaC via proteolytic cleavage of the ENaC γ-subunit (4). *In vivo*, skin-specific deletion of prostasin in mice resulted in early death after birth as a consequence of severe dehydration, demonstrating the involvement of prostasin in the epidermal barrier function(8). Animals harboring mutated prostasin genes [frizzy (*fr*) mouse and hairless (fr^{CR}) rats] exhibited reduced embryonic viability, skin defects, and decreased ENaC activity in the colon (9). Various functions of prostasin have been proposed for cellular growth, morphogenesis, and sodium absorption in various organs including the kidney and lung (4).

Reduced sodium reabsorption associated with downregulated ENaC in the distal colon is one of the causal factors of diarrhea in IBD patients (4). In the gastrointestinal tract, ENaC expression is limited to the epithelial cells of the distal colon and rectum, and aldosterone is a potent inducer of β and γ subunits of ENaC(10, 11). Increased expression

of ENaC in the colon induces sodium retention(7), but downregulation of ENaC leads to reduction of sodium reabsorption and leads to diarrhea(7). Previous studies have demonstrated a reduced expression of ENaC subunits in IBD patients(11, 12). Amasheh *et al.* reported that TNF-α selectively suppresses expression of β and γ subunits of ENaC and causes diarrhea in UC patients(11). Zeissig *et al.* also reported that sodium absorption via ENaC was strongly impaired even in the noninflamed colonic mucosa of CD patients due to reduced expression of the γ subunit of ENaC(12). Although dysregulation of ENaC has been reported to be involved in the pathophysiology of IBD, prostasin expression and its functional activity in IBD mucosa remain unclear.

The Toll-like receptors (TLRs) recognize highly conserved structural motifs known as pathogen-associated microbial patterns (PAMPs) widely expressed by microbial pathogens and initiate innate immune responses(13). Stimulation of TLRs leads to a variety of cellular responses including the pro-inflammatory cytokine production and effector cytokines that direct the development of antigen-specific acquired immunity(14). Among TLRs, TLR4 is predominantly activated by lipopolysaccharides (LPS), the major component of the outer membrane of gram-negative bacteria(14). TLR4 signaling has been reported to plays a crucial role in the pathophysiology of IBD(15).

In the present study, we investigated prostasin expression in the inflamed mucosa of IBD patients. We also developed mice lacking prostasin in the colonic epithelial cells to investigate the possible role of prostasin in the regulation of gut inflammation.

Material and methods

Tissue samples of IBD patients

Tissue samples of the sigmoid colon were obtained by biopsy under colonoscopy or

surgery from patients with UC and CD. The clinical activity of IBD was determined according to the colitis activity index for UC(16) and the CD activity index(17). Normal colonic samples were obtained by biopsy under screening colonoscopy for colon cancer. This project was approved by the ethics committee of the Shiga University of Medical Science (Permission number: 30-83), and written informed consents was obtained from all patients.

Animals and DSS colitis

PRSS8-floxed (*PRSS8*$^{lox/lox}$) mice were kindly provided by Prof. Kenichiro Kitamura (Faculty of Medicine, Yamanashi University, Japan) (18). Mice lacking the *PRSS8* gene in their intestinal epithelial cells (*PRSS8*$^{\Delta IEC}$ mice) were generated by crossing *PRSS8* $^{lox/lox}$ mice with transgenic mice that expressed Cre-recombinase driven by the villin promotor (*villin-Cre* mice: Jackson Laboratory, Bar Harbor, ME). All mice (six to eight weeks-old females) were housed under specific pathogen-free conditions. Mice were allowed free access to water and rodent chow (CE-2; CLEA Japan, Inc.). Experimental colitis was induced by the oral administration of 4% w/v dextran sodium sulfate (DSS: molecular weight 5000; Wako Pure Chemical Industries, Osaka, Japan) in tap water for 4 days followed by tap water for 3 days. Mice were divided into four groups of *PRSS8* $^{lox/lox}$ mice as a control group, *PRSS8*$^{\Delta IEC}$ mice, DSS-treated *PRSS8* $^{lox/lox}$ mice (*PRSS8* $^{lox/lox}$ DSS mice) and DSS-treated *PRSS8*$^{\Delta IEC}$ mice (*PRSS8*$^{\Delta IEC}$ DSS mice) (n=5 mice/group). This project was approved by the Research Center for Animal Life Science and Use Committee at the Shiga University of Medical Science (permission number: 2017-8-7).

Assessment of DSS induced colitis

The severity of colitis at distal colon was evaluated using the Disease Activity Index

(DAI) described in a previous report(19). Histological evaluations were performed according to a validated scoring system (20).

Isolation of colonic epithelial cells

Colonic epithelial cells were isolated from distal colon according to the method described previously(21).

Extraction of total RNA and real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). Real-time PCR was performed using the Lightcycler 480 System (Roche Applied Science, Penzberg, Germany) and SYBR Premix ExTaqII (TAKARA, Otsu, Japan). Expression of each target gene was normalized to β-actin and expressed as a ratio relative to the control group. The PCR primers used in this study are listed in Supplementary Table 1.

Immunohistochemistry and immunofluorescence

Immunohistochemical and immunofluorescence analyses were performed according to the method described previously (22). The used antibodies are listed in Supplementary Table2.

Extraction of protein and immunoblot analysis

Nuclear proteins were extracted from tissues or cells using the CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich, St. Louis, MO). Cytoplasmic proteins were obtained according to the method described previously (23). Samples were subjected to SDS-PAGE on a 4-20% gradient gel under reducing conditions and immunoblotting. The used antibodies are listed in Supplementary Table2.

Silencing of gene expression in cultured cells

The human colonic epithelial cell line HT-29 (ATCC, Manassas, VA) was transfected with small interference RNA (siRNA) specific for PRSS8 (Ambion, Carlsbad, CA) or

control siRNA using the Lipofectamine RNAiMAX regent (Invitrogen, Carlsbad, CA).

In vivo epithelial permeability assay

In vivo epithelial permeability was assessed using fluorescein isothiocyanate (FITC)-labeled dextran according to the method described previously (24). After 4h fasting, mice were orally administered FITC-dextran (0.44 mg/g body weight, MW 4000; Sigma-Aldrich Co., St Louis, MO). After 4h, blood was collected via cardiac puncture and was then centrifuged for 20 min to separate serum. The fluorescein intensity at 485-nm excitation wavelengths was measured.

Antibiotic treatment

PRSS8$^{\Delta IEC}$ mice were treated with a combination of ampicillin (1mg/ml: Nacalai Tesque, Kyoto, Japan), vancomycin (0.5mg/ml: Nacalai Tesque), neomycin sulfate (1mg/ml: Nacalai Tesque) and metronidazole (1mg/ml: Nacalai Tesque) in drinking water for 4weeks prior to DSS administration (25, 26).

Statistical analysis

Data were evaluated as means ± SEM. Statistical significance of the differences was determined by using one-way ANOVA with Bonferroni post hoc tests and the non-parametric Mann-Whitney U-test. $P < 0.05$ was considered statistically significant.

Results

Mucosal expression of prostasin (PRSS8) mRNA was evaluated using the real-time PCR method. As shown in Fig. 1A, prostasin mRNA expression was significantly decreased in the active mucosa of both UC and CD patients compared to normal and/ or inactive mucosa. This finding was confirmed by immunohistochemical staining (Fig. 1B). In normal colonic mucosa, prostasin was strongly expressed at the apical surfaces of colonic

epithelial cells. However, this staining was markedly decreased in active mucosa of UC and CD patients.

To investigate the role of prostasin in gut inflammation, we evaluated DSS colitis developed in mice in which prostasin is specifically deleted in intestinal epithelial cells (*PRSS8*$^{\Delta IEC}$ mice). In these mice, intestinal epithelial cell-specific prostasin deletion was confirmed at the mRNA and protein levels (Supplementary Fig. 1). Compared to *PRSS8* $^{lox/lox}$ mice, *PRSS8*$^{\Delta IEC}$ mice exhibited no changes in appearance, body weight gain or water intake. Some *PRSS8*$^{\Delta IEC}$ mice presented soft stool, but it was not severe. Under DSS load, body weight loss between days 5 to day 7 was significantly higher in *PRSS8*$^{\Delta IEC}$ mice than in control *PRSS8* $^{lox/lox}$ mice (Fig. 2A). Disease activity index on day 7 was significantly higher in DSS-treated *PRSS8*$^{\Delta IEC}$ mice than in DSS-treated *PRSS8* $^{lox/lox}$ mice (Fig.2B). There were no histological changes in colonic mucosa of *PRSS8*$^{\Delta IEC}$ mice (Fig. 2C), but DSS treatment induced more severe inflammatory changes, such as epithelial disruption and inflammatory cell infiltration, in *PRSS8*$^{\Delta IEC}$ mice than in *PRSS8* $^{lox/lox}$ mice (Fig. 2C). The histological colitis score was significantly higher in DSS-treated *PRSS8*$^{\Delta IEC}$ mice than in DSS-treated *PRSS8* $^{lox/lox}$ mice (Fig. 2D). Thus, targeted deletion of prostasin in intestinal epithelial cells exacerbated DSS colitis, suggesting a protective role of prostasin in gut inflammation.

TLR4 signaling has been reported to play a dominant role in the pathophysiology of IBD and DSS-colitis. In addition, it has been recently demonstrated that prostasin regulates TLR signaling in hepatocytes (18). Based on these findings, we addressed TLR4 expression in *PRSS8*$^{\Delta IEC}$ mice. Histological and immunoblot analyses demonstrated that TLR4 expression was weakly expressed in colonic epithelial cells of control *PRSS8* $^{lox/lox}$ mice, and this was strongly increased in *PRSS8*$^{\Delta IEC}$ mice (Fig. 3A and B), indicating that

prostasin is a negative regulator for TLR4 expression. This was supported by the observations in DSS-treated *PRSS8*$^{\Delta IEC}$ mice. DSS treatment enhanced TLR4 expression in colonic epithelial cells of *PRSS8* $^{lox/lox}$ mice and *PRSS8*$^{\Delta IEC}$ mice (Fig. 3A and B) and this effect was much stronger in *PRSS8*$^{\Delta IEC}$ mice (Fig. 3A and B).

NF-κB is a master transcription factor of inflammatory responses and its activation is closely associated with TLR4 signaling (27). In this study, we evaluated NF-κB activation by cytoplasmic accumulation of phosphorylated- (p-)IκBα and translocation of NF-κB p65 into the nucleus. Reflecting the changes in TLR4 expression in *PRSS8*$^{\Delta IEC}$ mice, immunoblot analyses showed that NF-κB activation in colonic epithelial cells was much stronger in DSS-treated *PRSS8*$^{\Delta IEC}$ mice than in DSS-treated *PRSS8*$^{lox/lox}$ mice (Fig. 3C). The mRNA expression of inflammatory cytokines (TNFα, IL-1β, IL-6 and CXCL1) which are induced through NF-κB activation was significantly higher in DSS-treated *PRSS8*$^{\Delta IEC}$ mice than in DSS-treated *PRSS8* $^{lox/lox}$ mice (Fig. 3D). These findings suggest that deletion of prostasin induced TLR4 expression in colonic epithelial cells and exacerbated colitis as a consequence of enhanced TLR4 signaling.

Interaction between PRSS8 and TLR4 expression was investigated *in vitro*. In the colonic epithelial cell line HT-29, PRSS8 mRNA expression was silenced by prostasin (PRSS8)-specific siRNA. As shown in Fig. 4A, immunoblot analysis revealed that prostasin protein expression was completely depleted in HT-29 cells transfected with PRSS8-specific siRNA. Prostasin deletion induced a marked enhancement of TLR4 protein expression (Fig. 4A left panel), although TLR4 mRNA was similarly expressed in control HT-29 cells and prostasin-depleted cells (Fig.4A right panel). Induction of TLR4 was supported by the findings of NF-κB activation in prostasin-depleted cells. LPS induced cytoplasmic accumulation of phosphorylated (p)-IκBα and translocation of NF-

κB p65 into the nucleus (Fig. 4B), and these responses were markedly enhanced in the cells transfected with prostasin-specific siRNA (Fig. 4B). Supporting these results, LPS-stimulated mRNA expression of inflammatory cytokines (TNFα, IL-1β, IL-6 and CXCL1) was significantly enhanced in the cells transfected with prostasin-specific siRNA compared to the cells transfected with control siRNA (Fig. 4C).

To confirm the possibility that LPS-stimulated TLR4 activation is involved in the exacerbation of DSS colitis in *PRSS8*$^{\Delta IEC}$ mice, *PRSS8*$^{\Delta IEC}$ mice were given a combination of broad-spectrum antibiotics in their drinking water for 4 weeks prior to DSS administration in order to deplete the commensal microflora. As shown in Fig. 5, there was no difference in body weight loss, disease activity or histological score between antibiotics-treated *PRSS8*$^{\Delta IEC}$ DSS mice and *PRSS8* $^{lox/lox}$ DSS mice. Thus, antibiotics treatment completely suppressed the exacerbation of DSS colitis in *PRSS8*$^{\Delta IEC}$ mice, suggesting a major role of commensal microflora-stimulated TLR-4 signaling in the exacerbation of DSS colitis in *PRSS8*$^{\Delta IEC}$ mice.

Increased mucosal permeability is one of the initiating and promoting factors for mucosal inflammation. Previous studies have demonstrated that DSS treatment induces a disruption of the tight junction and increases epithelial permeability (28, 29). So, we evaluated the changes in mRNA expression of tight junction proteins such as zonula occludens-1 (ZO-1), occludin, and claudin-1 in *PRSS8*$^{\Delta IEC}$ mice (29). As shown in Fig. 6A, there was no significant difference in the mRNA expression of tight junction proteins between *PRSS8* $^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice. DSS treatment induced a significant decrease in the mRNA expression of ZO-1, occludin and claudin-1 in both *PRSS8* $^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice, but there was no difference in mRNA expression of these genes between DSS-treated *PRSS8* $^{lox/lox}$ and DSS-treated *PRSS8*$^{\Delta IEC}$ mice. Furthermore, we evaluated the

changes in mucosal permeability using FITC-dextran. Deletion of prostasin did not affect mucosal permeability, since there was no difference in serum FITC-dextran levels between PRS*S* $^{lox/lox}$ mice and *PRSS8*$^{\Delta IEC}$ mice. Serum FITC-dextran levels were significantly increased in response to DSS treatment in both *PRSS8* $^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice, but there was no statistically significant difference between the two groups. These observations indicate that disruption of the tight junction and increased mucosal permeability were not involved in the exacerbation of DSS-colitis in *PRSS8*$^{\Delta IEC}$ mice.

Discussion

In the present study, we demonstrated several novel findings. The first is that prostasin expression is markedly reduced in the inflamed mucosa of UC and CD patients. The second is that mice with targeted deletion of prostasin in intestinal epithelial cells (*PRSS8*$^{\Delta IEC}$ mice) were more susceptible to DSS administration. The third is that deletion of prostasin lead to a marked increase in TLR4 expression and NF-κB activation in colonic epithelial cells. Finally, mucosal permeability in *PRSS8*$^{\Delta IEC}$ mice was comparable to that in control *PRSS8* $^{lox/lox}$ mice. These observations suggest the presence of dysregulated anti-inflammatory activity in the inflamed mucosa of IBD patients due to down-regulation of epithelial expression of prostasin.

The major process of sodium absorption in human distal colon is ENaC-mediated electrogenic sodium transport (5, 6). Previous studies have reported an impaired sodium absorption via altered ENaC in the inflamed mucosa of UC and CD patients, suggesting that impaired sodium absorption may be one of the factors that causes diarrhea in IBD patients (11, 12). Prostasin plays a crucial role in full activation of ENaC via proteolytic cleavage of the ENaC γ-chain (4), and deletion of prostasin in animals leads

to a marked disturbance of electrogenic sodium transport in the colon (7). Although there are a growing number of reports of basic research on prostasin (4), the pathophysiological role of prostasin in health and disease has not yet been fully elucidated. In this study, we demonstrated for the first time that prostasin expression is markedly reduced in the inflamed mucosa of IBD patients. This suggests that reduced prostasin expression may be one of the molecular mechanisms contributing to impaired ENaC-mediated electrogenic sodium absorption in IBD patients. In addition, these observations inspired us to investigate the pathological significance of reduced prostasin expression in IBD.

Keppner *et al.* previously reported that hairless (fr^{CR}) rats harboring a spontaneous mutation of the prostasin gene showed a reduced number of crypt and goblet cells and local inflammation with mild edema in the colon (30). These rats showed diarrhea but maintained mucosal permeability (30). Conversely, $PRSS8^{\Delta IEC}$ mice in this study exhibited normal appearance and normal colon. In $PRSS8^{\Delta IEC}$ mice, there were no histological signs of colitis and no abnormalities of intestinal permeability assessed by using FITC-dextran. These phenotypes of $PRSS8^{\Delta IEC}$ mice were consistent with a previous report using similar mice (7). Thus, it became clear that prostasin deletion in intestinal epithelial cells itself did not directly induced mucosal inflammation, although a previous study revealed that colonic ENaC-mediated sodium absorption is impaired in these mice (7).

DSS treatment induced colitis in both $PRSS8^{lox/lox}$ and $PRSS8^{\Delta IEC}$ mice. Colitis severity characterized by body weight loss, disease activity index and histological colitis score was significantly higher in $PRSS8^{\Delta IEC}$ mice than in $PRSS8^{lox/lox}$ mice, indicating a protective role of prostasin in DSS colitis. Recently, Uchimura *et al*. reported that prostasin regulates hepatic insulin sensitivity by modulating TLR4 signaling (18). They

demonstrated that a high-fat diet triggers the suppression of prostasin expression and increases the TLR4 level in the liver (18). Based on this report, we evaluated TLR4 expression in *PRSS8*$^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice. Mucosal expression of TLR4 was increased in *PRSS8*$^{\Delta IEC}$ mice compared to *PRSS8*$^{lox/lox}$ mice and in DSS-treated *PRSS8*$^{\Delta IEC}$ mice compared to DSS-treated *PRSS8*$^{lox/lox}$ mice, respectively. Reflecting increased TLR4 expression, NF-κB activation and the mRNA expression of pro-inflammatory cytokines were significantly increased in epithelial cells isolated from *PRSS8*$^{\Delta IEC}$ mice compared to those isolated from *PRSS8*$^{lox/lox}$ mice. Furthermore, silencing of the prostasin gene in the colonic epithelial cell line HT-29 using PRSS8-specific siRNA showed a direct suppressive effect of prostasin on TLR4 expression. These results indicate that prostasin has an anti-inflammatory effect via downregulation of TLR4 expression in colonic epithelial cells. It is likely that reduced expression of epithelial prostasin expression in IBD mucosa might be one of the promoting factors of mucosal inflammation via increased TLR4 signaling.

To evaluate a contribution of TLR4 signaling to exacerbation of DSS colitis in *PRSS8*$^{\Delta IEC}$ mice, *PRSS8*$^{\Delta IEC}$ mice were given a combination of broad-spectrum antibiotics in their drinking water for 2–4 weeks prior to DSS administration in order to deplete the commensal flora. This treatment has been reported to induce the sterility of the colons by bacteriologic analysis of fecal contents (26). As shown in Fig. 5, treatment with antibiotics completely abolished the exacerbation of DSS colitis in *PRSS8*$^{\Delta IEC}$ mice, indicating that an increased TLR4 signaling in response to commensal flora was a major factor contributing to exacerbation of colitis in *PRSS8*$^{\Delta IEC}$ mice.

The molecular mechanism underlying down-regulation of TLR4 by prostasin remains unclear. Uchiyama *et al*. previously demonstrated that in HepG2 cells

overexpressing TLR4, prostasin cleaves TLR4 by its serine protease activity and releases the ectodomain of TLR4 into the culture supernatant (18). A similar post-translational mechanism might be involved in our system, because TLR4 mRNA expression was equally detected in HT-29 cells with or without prostasin gene-silencing. Further experiments using intestinal epithelial cells overexpressing TLR4 may be helpful for identifying the mechanism of prostasin-mediated down-regulation of TLR4.

There were other possible reasons for the exacerbation of DSS-colitis in *PRSS8*$^{\Delta IEC}$ mice, including increased mucosal permeability after disruption of the epithelial barrier. To address this possibility, we checked mucosal mRNA expression of tight junction proteins. DSS treatment induced a significant decrease in mRNA expression of tight junction proteins in both *PRSS8*$^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice. However, there was no significant difference in decreased mRNA expression of these proteins between DSS-treated *PRSS8*$^{lox/lox}$ mice and DSS-treated *PRSS8*$^{\Delta IEC}$ mice. Furthermore, mucosal permeability was assessed by oral administration of FITC-dextran. DSS treatment induced a significant increase in mucosal permeability in both *PRSS8*$^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice, but there was no significant difference in increased mucosal permeability between DSS-treated *PRSS8*$^{lox/lox}$ mice and DSS-treated *PRSS8*$^{\Delta IEC}$ mice. These findings indicated that increased mucosal permeability did not contribute to exacerbation of DSS colitis in *PRSS8*$^{\Delta IEC}$ mice.

In conclusion, we found that expression of sodium absorption stimulator prostasin is markedly downregulated in the inflamed mucosa of IBD patients. From the observations in *PRSS8*$^{\Delta IEC}$ DSS mice, it became clear that prostasin has a strong anti-inflammatory effect via downregulation of TLR4 expression in colonic epithelial cells. So, a reduced prostasin expression in IBD mucosa appears to lead to a weakening of local

anti-inflammatory activity and may contribute to the persistence of mucosal inflammation. Finally, precise molecular mechanisms controlling prostasin expression at the inflammatory sites should be investigated in the future.

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Figure legends

Fig. 1 Prostasin expression in IBD mucosa. (A) The mRNA expression of prostasin in colonic mucosa of patients with IBD. Prostasin mRNA was evaluated in biopsy samples using real-time PCR. The mRNA expression of prostasin was calculated relative to the mRNA expression of β-actin and shown as fold increase compared to normal mucosa. The data are expressed as means ±SEM (normal n=14, UC inactive n=12, UC active n=13, CD inactive n=12, CD active n=13). Values not sharing a letter are significantly different (P<0.05). (B) Immunohistochemical analyses of prostasin expression.

Representative pictures in normal mucosa and inflamed mucosa of active UC and CD patients are presented. Control IgG staining are presented. Original magnification: ×200.

Fig. 2 DSS-colitis in mice lacking prostasin specifically in intestinal epithelial cells (*PRSS8*$^{\Delta IEC}$ mice). Experimental colitis was induced by oral administration of 4% w/v DSS (M.W. 5000) for 4 days and followed by 3 days water in control *PRSS8*$^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice. (A) Changes in relative body weight (n=5 of each group). Values not sharing a letter are significantly different (P<0.05). (B) Disease activity index. (C) Representative microscopic pictures of the distal colon on day 7 (original magnification×200). (D) Histological score. The data are shown as mean ± SEM (n=5). Values not sharing a letter are significantly different (P<0.05).

Fig. 3 TLR4 expression in control *PRSS8*$^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice.
(A) Immunohistochemical staining for TLR4 in control *PRSS8*$^{lox/lox}$ and *PRSS8*$^{\Delta IEC}$ mice. TLR4 (red fluorescence) and nucleus (DAPI, blue fluorescence). Representative pictures from four independent experiments. (B) Immunoblot analyses of TLR4 in the isolated colonic epithelial cells from mice. Representative pictures from three independent experiments. (C) Immunoblot analyses for phosphorylated (p)-IκBα in the cytoplasm and NF-κBp65 in the nucleus of isolated colonic epithelial cells from mice. GAPDH and laminin were used as a loading control. Representative picture from three independent experiments. (D) Real-time PCR analyses for mucosal mRNA expression of TNF-α, IL-1β, IL-6 and CXCL1. Relative mRNA expression of cytokines to that of β-actin was calculated and presented as fold increase. The data are expressed as means

± SEM (n= 5). Values not sharing a letter are significantly different (P<0.05).

Fig. 4 Effects of prostasin siRNA on TLR4 expression in HT-29 cells.

(A) (Left panel) Immunoblot analyses of TLR4 and prostasin (PRSS8) in HT-29 cells transfected with control and prostasin-specific siRNA. The pictures are representative of three independent experiments. (Right panel) TLR4 mRNA expression in HT-29 cells transfected with control or prostasin-specific siRNA. Relative mRNA expression of TLR4 to that of β-actin was calculated and presented as fold increase. The data are expressed as means ± SEM (n=5). N.S., not significant. (B) (Upper panel) Immunoblot analyses of phosphorylated(p)-IκBα in the cytoplasm of the cells stimulated with LPS for 15min. GAPDH was used as the loading control. (Lower panel) Immunoblot analyses for NF-κB p65 in the nucleus. Lamin A/C was used as a loading control in the nuclear protein. The pictures are representative of two independent experiments. (C) The mRNA expression for TNF-α, IL-1β, IL-6 and CXCL-1 was evaluated by real-time PCR. HT-29 cells were transfected with control or prostasin (PRSS8) siRNA and stimulated with LPS for 12h. Relative mRNA expression of cytokines to that of β-actin was calculated and presented as fold increase. The data are expressed as means ± SEM (n=5). Values not sharing a letter are significantly different (P < 0.05).

Fig.5 Effects of antibiotics treatment on the development of colitis in *PRSS8*$^{\Delta IEC}$ mice. (A) Relative body weight change. *PRSS8*$^{\Delta IEC}$ mice were given a combination of broad-spectrum antibiotics (see Materials and Methods section) in their drinking water for 4 weeks prior to DSS administration (n= 5). (B) Disease activity index on day7. N.S.: not significant. (C and D) Representative microscopic pictures of HE-staining of

the distal colon (original magnification×200) and histological score. (n= 5). N.S.: not significant.

Fig. 6. Changes in the mRNA expression of tight junction proteins and mucosal permeability using fluorescein isothiocyanate (FITC)-labeled dextran. (A) The mRNA expression for ZO-1, occludin, and claudin1 was evaluated by real-time PCR in colonic epithelial cells isolated from mice. Relative mRNA expression of tight junction proteins to that of β-actin was calculated and presented as fold increase. The data are expressed as means ± SEM (n=5). Values not sharing a letter are significantly different ($P < 0.05$). (B) Mice were orally administered FITC-dextran (4.4mg/10g body weight). After 4 hours serum fluorescence intensity was determined. The data are expressed as means ± SEM (n= 5). Values not sharing a letter are significantly different ($P<0.05$).