Interleukin-8 production via protease-activated receptor 2 in human esophageal epithelial cells

N. YOSHIDA[1], K. KATADA[2], O. HANDA[2], T. TAKAGI[2], S. KOKURA[2], Y. NAITO[2], N. MUKAIDA[3], T. SOMA[4], Y. SHIMADA[4], T. YOSHIKAWA[2] and T. OKANOUE[1]

[1]Molecular Gastroenterology and Hepatology, [2]Inflammation and Immunology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto; [3]Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Ishikawa; [4]Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan

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Abstract. Interaction between proteases and protease-activated receptor (PAR) 2 has been proposed to mediate inflammatory and immune response in the gastrointestinal tract. Recently, increase in interleukin (IL)-8 in the esophageal mucosa has been associated with the pathogenesis of esophagitis induced by reflux of gastric acids, bile acids or trypsin. The aims of the present study were to determine PAR2 expression in normal human esophageal epithelial cells (HEEC) and to evaluate the mediation of IL-8 production by trypsin-PAR2 interaction in HEEC. Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis revealed that PAR2 mRNA and protein were constitutively expressed in HEEC without upregulation by the stimulation with tumor necrosis factor α or trypsin. IL-8 was produced in a dose-dependent fashion when cells were stimulated with a PAR2 agonist such as trypsin or SLIGKV-amide. Blocking antibody to PAR2, camostat mesilate (a trypsin inhibitor), p-38 mitogen-activated protein kinase (MAPK) inhibitors or ERK1/2 inhibitors reduced IL-8 production from trypsin-stimulated HEEC. Mutation of the NFκB-, AP-1- and NF-IL-6-binding site on the IL-8 gene promoter abrogated the induction of luciferase activities stimulated with trypsin by 100, 80 and 50%, respectively. These results indicate that PAR2 activation in HEEC by trypsin induces NFκB- and AP-1-dependent IL-8 production in association with activation of p38 MAPK and ERK1/2, suggesting that esophageal inflammation may be induced by PAR2 activation via reflux of trypsin.

Correspondence to: Dr Norimasa Yoshida, Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
E-mail: nyoshida@koto.kpu-m.ac.jp

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Introduction

In recent years, the pathology of reflux esophagitis has been studied in terms of inflammation, and increased expression of interleukin (IL)-8, a potent activating factor of neutrophils, has been reported in esophageal biopsy specimens from patients with reflux esophagitis (1-3). We also showed that the expression of IL-8 mRNA in the esophageal mucosa was significantly correlated with the endoscopic grade of esophagitis or with inflammatory cell infiltration (4). In addition, we reported that cultured normal human esophageal epithelial cells (HEEC) could produce IL-8 in response to stimulation with various bile acids, especially conjugated bile acids under acidic conditions (5).

Reflux of proteases such as trypsin, as well as gastric acid and bile acids, has been reported to cause damage to the esophageal mucosa, but the mechanism of this injurious action has not yet been elucidated completely. It has been clarified that various proteases are not only involved in digestion of the extracellular matrix but also in the process of intracellular signaling via protease-activated receptors (PARs), thus having a variety of roles in inflammatory/immune responses (6,7). The PARs comprise seven transmembrane trimer G-protein-coupled receptors that are activated by specific proteases, and a total of 4 PAR family members (PAR 1-4) have already been cloned (7). Among them, PAR1, PAR3, and PAR4 are mainly activated by thrombin, while PAR2 is activated by trypsin, mast cell tryptase or blood clotting factors (VIIa, Xa). These peptidases activate PARs via cleavage of the extracellular N-terminal domain, which then enables the new N terminus to bind the receptor itself as a tethered ligand to activate G-protein-coupled signal transduction pathways (8). PARs can also be activated without proteolytic cleavage using five to six synthetic peptides corresponding to the new amino termini of the cleaved receptors (9). Platelets, vascular endothelial cells, and vascular smooth muscle cells all express PARs. In addition, it has recently been reported that PARs are expressed by epithelial cells of certain organs, including the lungs (bronchi), uterus, urinary bladder, and gastrointestinal tract, and are involved in the development of various pathologic conditions (10).

PAR1 and PAR2 are particularly abundant in the gastrointestinal tract. Studies have shown that PAR2 activation results in proinflammatory effects, including vasodilatation (11), edema (12), and leukocyte-endothelial interactions (12,13). It has also been suggested that luminal proteases activate PAR2 in the mouse colon to induce inflammation and disrupt the integrity of the intestinal barrier (14). However, the role of PAR2 in esophageal function has not been well investigated. In particular, the interaction between proteases such as trypsin and PAR2 in the pathogenesis of reflux esophagitis is poorly understood. Therefore, in the present study, we investigated PAR2 expression in esophageal epithelial cells and IL-8 production via the trypsin-PAR2 interaction.

Materials and methods

Cells. Normal HEEC from the cell line established by Shimada *et al* were used (5,15,16). They were plated in tissue culture dishes and grown in keratinocyte serum-free medium containing 2.5 μg of epidermal growth factor and 25 μg bovine pituitary extract (Gibco BRL, Rockville, MD). They were seeded in 96-well plates or 10-cm tissue culture plates and utilized when they became confluent.

Reagents. Thrombin (human plasma) as a natural PAR1 agonist and trypsin (porcine pancreas) as a natural PAR2 agonist were purchased from Sigma-Aldrich Co. (St. Louis, MO). A synthetic PAR2 agonist (SLIGKV-NH_2) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Camostat mesilate, a serine protease inhibitor, was kindly provided by Ono Pharmaceuticals (Osaka, Japan).

Assessment of cytotoxicity. HEEC were incubated in 96-well plates for 18 h with either TNF-α (10 ng/ml), trypsin (0.1 nM), thrombin (10 U/ml) or SLIGKV-NH_2 (200 μM), and then the cell viability was assessed using the trypan blue exclusion test.

RT-PCR for PAR2 mRNA. Cells were plated in 10-cm Petri dishes. After reaching confluence, the cells were incubated with TNF-α (10 ng/ml), thrombin (10 U/ml), or trypsin (0.1 nM) for 3 h. Total cellular RNA was extracted by the acid guanidinium phenol chloroform method using an Isogen kit (Nippon Gene, Tokyo, Japan). The reverse transcription product (1 ml) was added to 3-mM concentrations of primers for PAR2 and GAPDH (as an internal standard), in a 50-ml solution containing 0.5 U of TaqDNA polymerase (Takara Biochemicals, Shiga, Japan). The primers were as follows: for human PAR2, sense primer 5'-GTTGATGGCACATCC CACGTC-3' and antisense primer 5'-GTACAGGGCATAG ACATGGC-3'; for GAPDH, sense primer 5'-ACCACAGT CCATGCCATCAC-3' and antisense primer 5'-TCCACCA CCCTGTTGCTGTA-3'. The PCR settings for the thermal profile were as follows: 30 cycles of 1 min at 94˚C, 1 min at 64˚C (60˚C for GAPDH), and 1 min at 72˚C. Then electrophoresis of the products was performed on 2.5% agarose gel.

Western blot analysis for PAR2. Cells were stimulated with TNF-α (10 ng/ml), thrombin (10 U/ml), or trypsin (0.1 nM) for 8 h, and then lysed in 150 μl of cell lysis buffer. After harvesting of the lysate with a cell scraper and centrifugation, the supernatant was collected. Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of extracts (15 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Boston, MA). The membrane was blocked with 3% nonfat dried milk in phosphate-buffered saline (PBS) for 30 min and then washed with PBS. Subsequently the membrane was incubated with rabbit IgG antibody to PAR2 diluted 1:1000 (H-99; Santa Cruz Biotechnology Inc.) overnight at 4˚C. Then the membrane was washed with PBS and incubated with anti-rabbit IgG secondary antibody diluted 1:2000 (Amersham, Buckinghamshire, UK) for 30 min at room temperature. The immunocomplexes on the membrane were visualized by treatment with a commercial kit (ECL; Amersham) according to the manufacturer's recommendations.

IL-8 protein production by PAR2 agonists. HEEC were seeded into a 96-well plate. HEEC were stimulated with trypsin (0.05 nM, 0.1 nM) as a natural PAR2 agonist, a synthetic PAR2 agonist peptide (SLIGKV-NH_2; 50, 100, 200 μM) or a control peptide (LSIGKV-NH_2; 50, 100, 200 μM). After incubation for the indicated time, levels of IL-8 protein in culture supernatant were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Bio Source International Inc., Camarilla, CA).

Effects of anti-PAR2 antibody or protease inhibitor on IL-8 production. The role of PAR2 on IL-8 production by HEEC was investigated using a blocking antibody against the amino-terminal cleavage region of PAR2 (SAM11; Santa Cruz Biotechnology Inc.). HEEC were grown to confluence in 96-well plates. Two hours after pre-incubation with anti-PAR2 blocking antibody (20 μg/ml), trypsin (0.1 nM) was added. Four hours later, IL-8 protein in the culture supernatant was measured by ELISA. As a control, mouse immunoglobulin (Santa Cruz Biotechnology Inc.) was used instead of blocking antibody. In addition, after HEEC were pretreated for 1 h with camostat mesilate (0.1-10 μM), a serine protease inhibitor, trypsin (0.1 nM) was added and the incubation was continued for another 4 h. Then the supernatant was harvested and the IL-8 level was determined.

Effect of the mitogen-activated protein kinase (MAPK) inhibitors on IL-8 production. The role of MAPK on IL-8 production from HEEC treated with trypsin was investigated using a p38 MAPK inhibitor (SB203580, Sigma Chemical Co., St. Louis, MO) and an extracellular regulated kinase 1/2 (ERK1/2) inhibitor (UO126; Sigma Chemical Co.). After pre-incubation with each inhibitor for 1 h, trypsin (0.1 nM) was added followed by incubation for 4 h. Subsequently, the supernatant was harvested and the IL-8 level was determined by ELISA.

Luciferase assay. The 5'-flanking region of the IL-8 gene spanning from bp -133 to +44 was subcloned into a luciferase expression vector as previously described (17). Site-directed mutagenesis of the IL-8 activator protein-1 (AP-1), nuclear

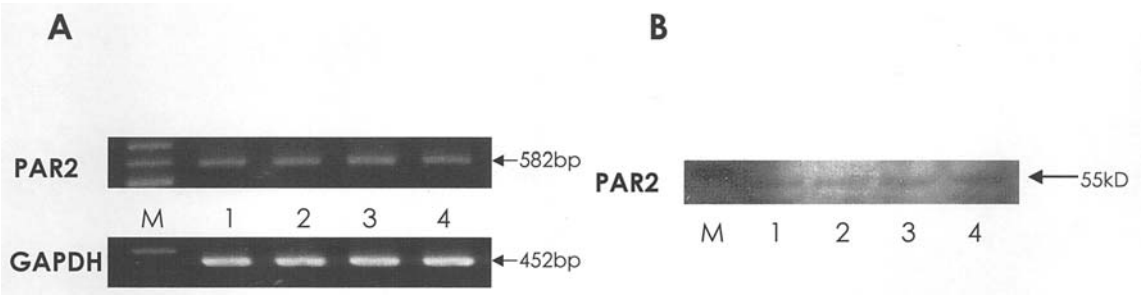

Figure 1. Protease-activated receptor 2 (PAR2) expression on normal human esophageal epithelial cells (HEEC). (A) RT-PCR. After HEEC were incubated with TNF-α, thrombin or trypsin for 3 h, total RNA was extracted from HEEC, and PAR2 mRNA expression was assessed by RT-PCR. (B) Western blotting. After HEEC were incubated with TNF-α, thrombin or trypsin for 8 h, PAR2 protein expression was evaluated by Western blot analysis. Lane 1, unstimulated; lane 2, TNF-α (10 ng/ml); lane 3, thrombin (10 U/ml); and lane 4, trypsin (0.1 nM).

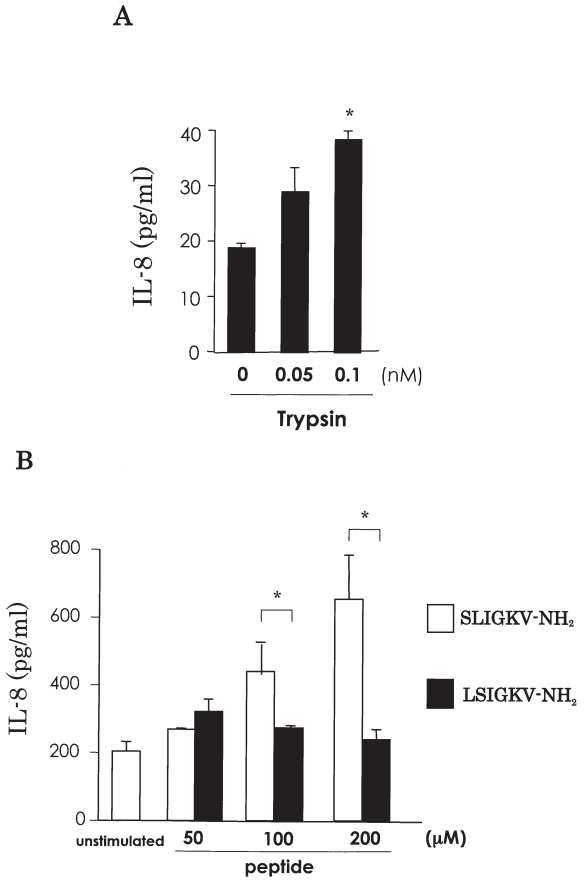

Figure 2. Interleukin-8 (IL-8) production in HEEC stimulated by PAR2 agonists. HEEC were incubated with PAR2 agonists, trypsin or SLIGKV-NH_2, and IL-8 protein levels in the culture supernatants were determined using enzyme-linked immunosorbent assay. (A) Stimulation with trypsin (4 -h incubation), and (B) stimulation with SLIGKV-NH_2 (18-h incubation). Each value represents the mean ± SE of three independent experiments performed in duplicate. *P<0.05 compared to the unstimulated group without trypsin (A) and to the control peptide (LSIGKV-NH_2) group (B).

factor for interleukin-6 expression (NF-IL6), and nuclear factor-κB (NF-κB) binding sites was carried out as previously described (17,18). For the luciferase assay, $3x10^6$ HEEC were transfected with 10 mg of each luciferase vector along with 2 mg of ß-galactosidase expression vector as an internal control, using Lipofectamine reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. After 24 h, the transfected cells were divided into two parts, and trypsin (0.1 nM) was added separately to one part. After an additional 8 h of incubation, cell lysates were prepared using a Pica Gene kit (Tokyo Ink Co., Tokyo, Japan), and their protein concentrations were measured by using a Bio-Rad protein assay kit, with bovine serum albumin as a standard, according to the manufacturer's instructions. The light intensities were measured on 20 μg of cell lysates by using a Pica Gene kit and a model BLR-301 luminescence reader (Aloka Co., Ltd., Tokyo, Japan).

Statistical analysis. Data in each experiment are expressed as the mean ± standard error (SE). Statistical analysis was evaluated using an analysis of variance (ANOVA) followed by Fischer's protected least significant difference test (Fischer's PLSD). A level of $P<0.05$ was considered statistically significant.

Results

Cytotoxicity. The cell viability was >95% for HEEC stimulated by TNF-α, thrombin, trypsin or PAR2 agonist (SLIGKV-NH_2) (data not shown).

PAR2 expression in HEEC. The mRNA expression of PAR2 was constitutively detected on unstimulated HEEC by RT-PCR, but the level of PAR2 expression showed no appreciable increase when the cells were stimulated with TNF-α (10 ng/ml), thrombin (10 U/ml) or trypsin (0.1 nM) (Fig. 1A). The presence of PAR2 protein on unstimulated HEEC was confirmed by Western blot analysis. The stimulation of HEEC with TNF-α, thrombin or trypsin had no influence on the level of PAR2 protein (Fig. 1B).

IL-8 production in HEEC stimulated by trypsin (Fig. 2). IL-8 production increased dose-dependently when the cells were stimulated with trypsin, an endogenous PAR2 agonist, for 4 h (Fig. 2A). In addition, IL-8 production also increased dose-dependently when the cells were stimulated with SLIGKV-NH_2, a synthetic PAR2 agonist, for 18 h, while IL-8 production showed no significant increase when the cells were stimulated with LSIGKV-NH_2, a control peptide for a synthetic PAR2 agonist (Fig. 2B).

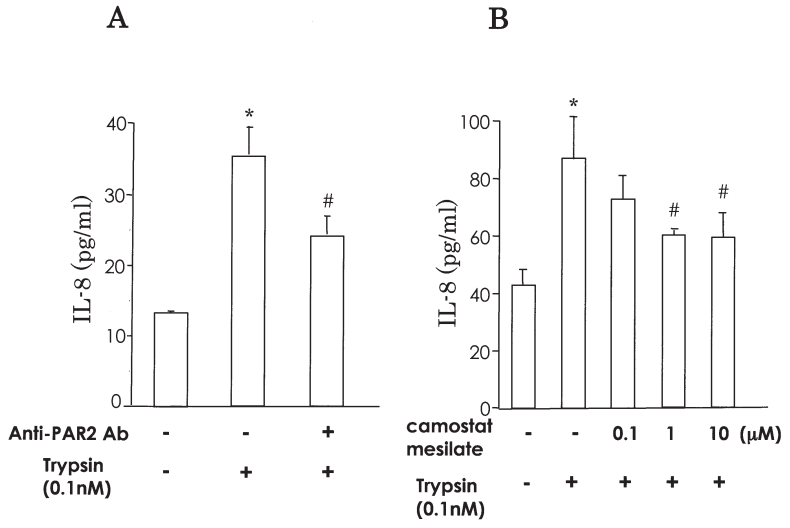

Figure 3. Effect of blocking antibody to PAR2 (A) and camostat mesilate (B) on IL-8 production from trypsin-stimulated HEEC. (A) After HEEC were pretreated with blocking antibody to PAR2 (20 μg/ml) for 2 h, they were stimulated by trypsin (0.1 nM). Four hours later, IL-8 protein in the supernatants was assessed by ELISA. (B) After HEEC were pretreated with camostat mesilate (0.1-10 μM), a protease inhibitor, for 1 h, they were stimulated by trypsin (0.1 nM). Four hours later, IL-8 protein in the supernatants was assessed by ELISA. Each value represents the mean ± SE of three independent experiments performed in duplicate. *P<0.05 compared to the unstimulated group without trypsin or camostat mesilate. #P<0.05 compared to the trypsin group without anti-PAR2 antibody (A) or camostat mesilate (B).

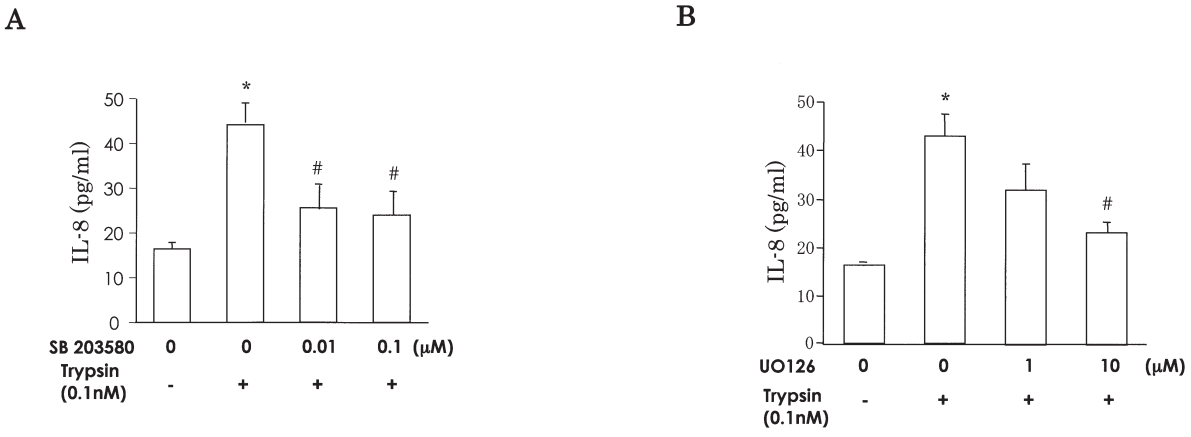

Figure 4. Effect of the mitogen-activated protein kinase (MAPK) inhibitors on IL-8 production in HEEC. After HEEC were preincubated with SB203580 (p38 inhibitor) or UO126 (ERK1/2 inhibitor) for 1 h, they were stimulated by trypsin (0.1 nM). The IL-8 protein concentration in the culture supernatants was determined by ELISA 4 h after stimulation. (A) Pretreatment with SB203580 and (B) pretreatment with UO126. Each value represents the mean ± SE of three independent experiments performed in duplicate. *P<0.05 compared to the unstimulated group without trypsin. #P<0.05 compared to the trypsin group without MAPK inhibitor.

Effect of blocking antibody to PAR2 and protease inhibitor. As shown in Fig. 3A, IL-8 production from HEEC stimulated with trypsin was significantly inhibited by the pretreatment with blocking antibody against PAR2. In addition, pretreatment with camostat mesilate at concentrations of 1-10 μM also significantly inhibited the IL-8 production induced by trypsin (Fig. 3B).

Effect of MAPK inhibitors on IL-8 production. The trypsin-induced production of IL-8 protein by HEEC was significantly suppressed by pretreatment with p38 MAPK inhibitor (Fig. 4A) and ERK1/2 inhibitor (Fig. 4B) in a dose-dependent manner.

Luciferase activity (Fig. 5). Introduction of a mutation at the NF-κB-binding sites on the IL-8 gene promoter almost completely inhibited the increase of luciferase activity, while the increase was inhibited by ~80% after introduction of the AP-1 mutation and ~50% by the NF-IL-6 mutation.

Discussion

The present study revealed that PAR2 was constitutively expressed on normal HEEC. It has been shown that PARs, especially PAR1 and PAR2, are widely distributed in the gastrointestinal organs, and are involved in various processes, including exocrine secretion from the salivary glands, production of gastrointestinal mucus and pepsin, control of gastric and intestinal peristalsis, secretion of pancreatic juice, and regulation of ion transfer across the small bowel mucosa (19). However, in the esophagus, though PAR1 and PAR4

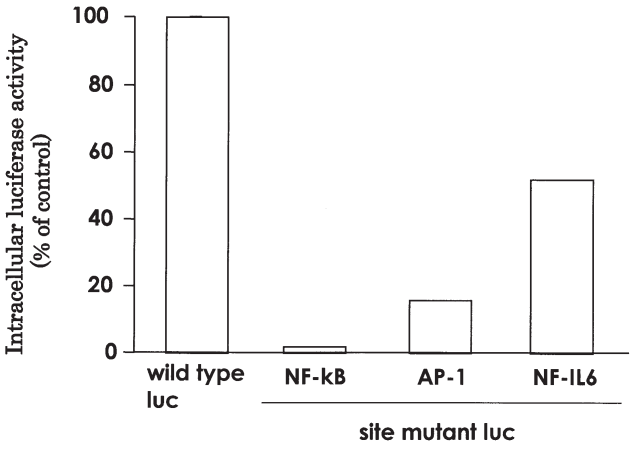

Figure 5. IL-8 gene transcription through activating NF-κB, AP-1 and NF-IL6. HEEC were transfected with various luciferase expression vectors and were stimulated with trypsin (0.1 nM). Intercellular luciferase activities were determined 8 h after stimulation. Data are presented as a percentage of the control value with wild-type luciferase vector. Representative results from two independent experiments are shown.

have been demonstrated to be expressed in the isolated rat esophageal muscularis mucosae (20), this is the first report demonstrating constitutive expression of PAR2 mRNA and protein in esophageal epithelial cells.

The present study also showed that PAR2 activation by trypsin or SLIGKV-NH_2, a PAR2 agonist, induced IL-8 production in a dose-dependent manner. In addition, trypsin-mediated IL-8 production from HEEC was significantly inhibited by pretreatment with a blocking antibody to PAR2 or camostat mesilate, a trypsin inhibitor. These results suggest that reflux of duodenal fluid containing trypsin can induce PAR2-dependent IL-8 production from esophageal epithelial cells, followed by esophageal inflammation. It has been reported that PAR2 activation induces production of IL-1ß, IL-8, and ICAM-1 by lung epithelial and umbilical vein endothelial cells (21). In addition, Cenac *et al* (14) reported that intracolonic administration of a PAR2 agonist induced the rapid development of colonic inflammation with increased neutrophil infiltration and an elevation of T-helper cell type 1 cytokines. Taken together with our present findings, these results suggest that PAR2 activation associated with the localized increase of serine proteases can induce cytokine-related inflammation.

In contrast, it has also been reported that PAR2 plays an important role in the anti-inflammatory reaction and cyto-protective effects. Fiorucci *et al* (22) have revealed that repeated subcutaneous administration of PAR2 agonist prevents the development and induces the healing of T helper cell type 1-mediated experimental colitis in mice. In addition, it has been clarified that administration of PAR2 agonist promotes the secretion of gastric mucus mediated by the stimulation of capsaicin-sensitive sensory nerves (23), and that PAR2 exhibits a neural/prostaglandin-independent inhibitory effect on the secretion of gastric acid (24). Though the present study demonstrated that PAR2 activation had a proinflammatory effect in esophageal epithelial cells, further investigation will be needed to clarify the anti-inflammatory effect of PAR2 in the development of esophageal inflammation.

In clinical practice, camostat mesilate is used for the treatment of reflux esophagitis after gastrectomy (25), although the mechanism by which trypsin induces esophagitis has not been completely elucidated. In the present study, trypsin-stimulated IL-8 production via PAR2 was inhibited by camostat mesilate, suggesting that treatment with a protease inhibitor can reduce the esophageal inflammation associated with PAR2-dependent IL-8 production by inhibiting trypsin activity. This result is consistent with our recent evidence that camostat mesilate reduces esophageal inflammation with an increased level of cytokine-induced neutrophil chemoattractant-1 (CINC-1), which is relative to IL-8 chemokine in humans (26).

The mechanism of intracellular signaling after activation of PAR2 by trypsin or a synthetic agonist of PAR2 has not been completely elucidated. However, it is known that both trypsin and PAR2 agonist peptide stimulate inositol tri-phosphate formation and Ca^+ mobilization in numerous cell types, consistent with coupling to the heterotrimeric G-proteins G_q/G_{11} and phospholipase C isoforms (10). Recently, some papers have demonstrated that PAR2 agonists induced p38 MAPK and ERK1/2 phosphorylation and activation (27,28). Coupling to this pathway would be consistent with linkage to a number of proinflammatory responses in target cell types (10). In the present study using selective inhibitors, we showed that p38- and ERK1/2-MAPK are involved in the PAR2 signaling mechanism of IL-8 production from HEEC. In addition, our recent study revealed that p38 MAPK is implicated in the IL-8 production by HEEC exposed to bile acids (5). These results suggest the importance of MAPK activation in the PAR2-signaling pathways.

IL-8 gene transcription requires the activation of NF-κB in combination with AP-1 or NF-IL6, depending on the types of cells (5). The present results of the luciferase assay indicated that the main transcription factor involved in IL-8 production was NF-κB, and that AP-1 and NF-IL6 were also involved to a lesser degree, as reported for the production of IL-8 in HEEC stimulated by cytokines or bile acids (5). With respect to the treatment of reflux esophagitis, these findings suggest that PAR2 antagonists and selective NF-κB inhibitors currently under development may be useful for reducing trypsin-stimulated production of IL-8.

In summary, the results of the present study confirm that PAR2 is expressed in normal human esophageal epithelial cells and suggest that both trypsin and SLIGKV-NH_2 may activate esophageal epithelial cells via PAR2 to induce NF-κB- and AP-1-dependent IL-8 production, thus playing a role in the onset of esophagitis. In addition, our recent data indicate that increased IL-8 expression in the esophageal mucosa is closely associated with neutrophil-dependent esophageal inflammation in humans and rats (4,29). Taken together, this evidence suggests that IL-8, synthesized by the esophageal mucosal epithelial cells in response to refluxed materials including trypsin and bile acids, induces neutrophil activation and infiltration, leading to mucosal damage. This may aid in the further elucidation of the mechanism of inflammation in the esophageal mucosa in GERD, and the development of new therapeutic modalities for this disease.

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