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Activation of Human Oral Epithelial Cells by Neutrophil Proteinase 3 Through Protease-Activated Receptor-21

Akiko Uehara,* Shunji Sugawara,2* Koji Muramoto, † and Haruhiko Takada*

Proteinase 3 (PR3), a 29-kDa serine proteinase secreted from activated neutrophils, also exists in a membrane-bound form, and is suggested to actively contribute to inflammatory processes. The present study focused on the mechanism by which PR3 activates human oral epithelial cells. PR3 activated the epithelial cells in culture to produce IL-8 and monocyte chemoattractant protein-1 and to express ICAM-1 in a dose- and time-dependent manner. Incubation of the epithelial cells for 24 h with PR3 resulted in a significant increase in the adhesion to neutrophils, which was reduced to baseline levels in the presence of anti-ICAM-1 mAb. Activation of the epithelial cells by PR3 was inhibited by serine proteinase inhibitors and serum. The epithelial cells strongly express protease-activated receptor (PAR)-1 and PAR-2 mRNA and weakly express PAR-3 mRNA. The expression of PAR-2 on the cell surface was promoted by PR3, and inhibited by cytochalasin B, but not cycloheximide. PR3 cleaved the peptide corresponding to the N terminus of PAR-2 with exposure of its tethered ligand. Treatment with PR3, an agonist for PAR-2, and a synthetic PAR-2 agonist peptide induced intracellular Ca2+ mobilization, and rendered cells refractory to subsequent stimulation with PR3 and vice versa. The production of cytokine induced by PR3 and the PAR-2 agonist peptide was completely abrogated by a phospholipase C inhibitor. These findings suggest that protease-activated receptor-2 (PAR-2) and protease-activated receptor-3 (PAR-3) mediate the inflammatory response to PR3 stimulation.

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1 Abbreviations used in this paper: PAR, protease-activated receptor; α1-AT, α1-antitrypsin; Boc-Ala-ONp, Boc-Ala-p-nitrophenyl ester; Cat G, cathepsin G; CDS, cell dissociation solution; EM, extracellular medium; GCF, gingival crevicular fluid; HLE, human leukocyte elastase; MCP-1, monocyte chemoattractant protein-1; PLC, phospholipase C; PR3, proteinase 3.
express a 24-kDa precursor form of IL-18, and that human neutrophil PR3 induced the secretion of a bioactive IL-18 from the epithelial cells in combination with LPS after priming with IFN-γ (27). We extended the investigation of the underlying mechanism by which PR3 activates the epithelial cells, and in the present study, we obtained the evidence for the first time that PR3 by itself could activate the epithelial cells in culture to induce production of IL-8 and MCP-1 and expression of ICAM-1 via the PAR-2 pathway.

Materials and Methods

Reagents

Purified human neutrophil PR3 was obtained from HyTest (Turku, Finland) and from Elastin Products (Owensville, MO). Purified HLE, Cat G, phospholipase C (PLC) inhibitor U73122, and control compound U73343 were obtained from Calbiochem-Novabiochem (La Jolla, CA). The purity of three enzymes (PR3, HLE, and Cat G) was >95% by SDS-PAGE, according to the manufacturer, and further confirmed by Western blotting using anti-human PR3 (Elastin Products), HLE, and Cat G (Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal Abs. The result showed that no cross-reactivity was observed in each preparation (data not shown). Human rIL-1α and rTNF-α were supplied by Dainippon Pharmaceutical (Osaka, Japan). Boc-Ala-p-nitrophenyl ester (Boc-Ala-ONp) was purchased from Bachem (Bubendorf, Germany). A low toxic serine proteinase inhibitor, Pefabloc SC and α-antitrypsin (α-AT), and FCS for 30 min at 37°C before use. Cultivation was conducted in triplicate, and levels of IL-8 and MCP-1 in the supernatants were determined using OptEIA ELISA kits (BD Pharmingen, San Diego, CA). The concentrations of the cytokines in the supernatants were determined using the Softmax data analysis program (Molecular Devices, Menlo Park, CA).

Flow cytometry

Flow cytometric analyses were performed using a FACScan cytometer (BD Biosciences, Mountain View, CA), as described (32). Oral epithelial cells were pretreated with or without cycloheximide (Bachem, Switzerland) to inhibit the enzymatic activity of PR3, it was preincubated with serine proteinase inhibitors, Pefabloc SC and α-antitrypsin (α-AT), and FCS for 30 min at 37°C before use. Cultivation was conducted in triplicate, and levels of IL-8 and MCP-1 in the supernatants were determined using OptEIA ELISA kits (BD Pharmingen, San Diego, CA). The concentrations of the cytokines in the supernatants were determined using the Softmax data analysis program (Molecular Devices, Menlo Park, CA).

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5'-AAGGAAGGCTGGAAGAGTGC-3' (27). The primers for IL-8, MCP-1, ICAM-1, PAR-1, PAR-2, PAR-3, PAR-4, and GAPDH were constructed to generate fragments of 422, 257, 243, 708, 1,066, 858, 725, and 527 bp, respectively. Cycling conditions were as follows: IL-8, 25 cycles at 94°C for 1 min, 63°C for 1 min, and 72°C for 3 min; MCP-1, ICAM-1, and GAPDH, 35 cycles at 94°C for 1.5 min, 60°C for 1 min, and 72°C for 3 min; PAR-1, 34 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and PAR-2, PAR-3, and PAR-4, 36 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min. Amplified samples were visualized on 2% agarose gels stained with ethidium bromide and photographed under UV light.

Analysis of peptide cleavage
A peptide corresponding to a region spanning the cleavage site of the PAR-2, residues 32–45 (5’SSKGRSLIGKVDGTT’3) (37), was synthesized by Takara. The peptide (200 μM) was incubated with proteins for 30 min at 37°C in PBS. Each digest was separated by reversed-phase HPLC on a Wakosil 5C4-200 column (5 mm, 4.6 × 250 mm) (WAKO, Osaka, Japan) using a linear gradient from 0 to 30% acetonitrile in 0.1% trifluoroacetic acid, and the amino acid sequences of peptide fragments were analyzed by a gas phase protein sequencer (PSQ-1; Shimadzu, Kyoto, Japan) and a matrix-assisted laser desorption ionization time of flight mass spectrometry (Kompact MALDI I; Shimadzu), according to a previously described procedure (38).

Calcium mobilization
Confused oral epithelial cells were collected by nonenzymatic CDS, washed twice with PBS, and suspended at 2 × 10⁶ cells/ml in an extracellular medium (EM; 121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 6 mM NaHCO₃, 5.5 mM glucose, 25 mM HEPES, 0.1% w/v BSA, pH 7.3). Cells were loaded with 1 μM Fura 2-AM with shaking for 30 min at room temperature. After being washed with EM, cells were resuspended in EM and incubated for 30 min at room temperature. After another wash with EM, loaded cells suspended at 2 × 10⁶ cells/ml were incubated in EM without BSA were transferred to stirred quartz cuvettes in a CAF-100 spectrophotometer (Jasco, Tokyo, Japan) at 37°C. Fura 2 fluorescence was measured at 340 and 380 nm excitation and 510 nm emission. The ratio of the fluorescence at the two excitation wavelengths, which is proportional to [Ca²⁺]ᵢ, was calculated.

Coculture of oral epithelial cells with stimulated neutrophils
Confused HSC-2 cells in 96-well plates were cocultured with the indicated number of purified neutrophils in 200 μl extracellular medium in the presence or absence of various concentrations of PR3 for 24 h. Cytospin cultures were also performed in the presence or absence of 10^{-3}M Pefabloc SC, α₁-AT, and FCS for 24 h. After the incubation periods of 24 h, supernatants in the supernatants were measured by ELISA.

Data analysis
All experiments in this study were performed at least three times to confirm the reproducibility of the results. In each experiment, values are represented as means ± SD of triplicate assays. The statistical significance of differences between the two means was analyzed by one-way ANOVA, using the Bonferroni or Dunn method, and p-values less than 0.05 were considered significant.

Results
Effect of PR3 on the production of IL-8 and MCP-1 by oral epithelial cells
We first examined the effect of PR3 on the production of IL-8 and MCP-1 by oral epithelial cell lines, HSC-2 and KB. Incubation of both cell lines in the presence of various concentrations of PR3 for 24 h resulted in a dose-dependent increase in IL-8 and MCP-1 (Fig. 1A). PR3 at 5–10 μg/ml was most effective in stimulating the production. Incubation of HSC-2 cells with 10 μg/ml (345 ng/ml) PR3 resulted in a time-dependent increase in the production of IL-8 and MCP-1 (Fig. 1B). A significant increase in production was observed from 8-h incubation, and a marked increase at 24 and 48 h. In contrast to PR3, two other neutrophil serine proteinases, HLE and Cat G, showed only marginal activity in oral epithelial cells as assessed by MCP-1 production (Fig. 1C), which was consistent with our recent observation (27). The PR3 from the two different sources showed the same results (Fig. 1D). IL-8 mRNA was already expressed in untreated cells, but MCP-1 mRNA was not expressed in untreated cells, and the expression of IL-8 and MCP-1 mRNA was increased and induced by PR3, respectively (Fig. 1E). IFN-γ (1000 IU/ml) and IL-1α (10 ng/ml) were used as

FIGURE 1. Effect of PR3 on the production of IL-8 and MCP-1 by human oral epithelial cells. Confluent HSC-2 and KB were incubated for 24 h in the presence of various concentrations of PR3 (A), or incubated in the presence (+) or absence (−) of PR3 (10 μg/ml) for the time indicated (B). C, Confluent HSC-2 cells were incubated in the presence of various concentrations of PR3, HLE, and Cat G for 24 h. D, Confluent HSC-2 cells were incubated in the presence of various concentrations of PR3 from HLE and Cat G, showed only marginal activity in oral epithelial cells. Confluent HSC-2 and KB were incubated for 8 h with melittin (lane 2), 10 μg/ml (lane 3), or 1000 IU/ml IFN-γ for 24 h. E, The expression of IL-8 and MCP-1, and GAPDH mRNA was analyzed by RT-PCR. Water control was loaded in lane 1. F, Confluent primary oral epithelial cells were incubated for 24 h in the presence of various concentrations of PR3. Concentrations of IL-8 and MCP-1 in the culture supernatants were determined by ELISA. E, Confluent HSC-2 and KB were incubated for 8 h with medium alone (lane 2), 10 μg/ml PR3 (lane 3), or 1000 IU/ml IFN-γ for IL-8 or 10 ng/ml IL-1α for MCP-1 (lane 4), and the expression of IL-8, MCP-1, and GAPDH mRNA was analyzed by RT-PCR. Water control was loaded in lane 1. F, Confluent primary oral epithelial cells were incubated for 24 h in the presence of various concentrations of PR3. Concentrations of IL-8 and MCP-1 in the culture supernatants were determined by ELISA. *p < 0.05, and **p < 0.01 compared with the respective control (medium alone). The results presented were representative of three different experiments demonstrating similar results.
a positive control for IL-8 and MCP-1 mRNA expression, respectively. Oral epithelial cells in primary culture also produce IL-8 and MCP-1 in response to PR3, although the cytokine levels were lower than those in the epithelial cell lines (Fig. 1F).

PR3-induced ICAM-1 mediates adhesion of oral epithelial cells to neutrophils

We next examined the effect of PR3 on the expression of an adhesion molecule, ICAM-1, on HSC-2 cells by flow cytometry. Assessment of the surface expression of ICAM-1 on the untreated cells revealed no reactivity (less than 2%), but the cells treated with increasing concentrations of PR3 for 24 h showed a dose-dependent increase of ICAM-1 expression, with a plateau in the level of ICAM-1 expression reached at 1 μg/ml PR3 (Fig. 2A). TNF-α was used as a positive control and also enhanced ICAM-1 expression. Incubation of HSC-2 cells with 10 μg/ml PR3 resulted in a time-dependent increase in the expression of ICAM-1 (Fig. 2B). The expression was up-regulated from 8-h incubation, then increased linearly until 48 h, at which point almost all the cells expressed ICAM-1. A representative FACS profile of ICAM-1 expression on untreated cells was shown in Fig. 2D. TNF-α (5 ng/ml) was used as a positive control. We then examined whether the ICAM-1 induced by PR3 in HSC-2 cells is involved in the adhesion to neutrophils. After the incubation of HSC-2 cells with 10 μg/ml PR3 for 24 h, a 9.1-fold increase in the adhesion of neutrophils was observed as compared with the cells incubated in the medium alone (Fig. 2E). TNF-α (5 ng/ml), as a positive control, also significantly promoted the adhesion to neutrophils and to HSC-2 cells. Furthermore, pretreatment of HSC-2 cells with anti-ICAM-1 mAb resulted in a complete reduction of adhesion of neutrophils, indicating that the ICAM-1 expressed on oral epithelial cells by PR3 was a major factor in the adhesion to neutrophils. KB showed the same results (data not shown). The findings shown in Figs. 1 and 2 indicate that PR3 activates oral epithelial cells, consequently inducing production of IL-8 and MCP-1, and expression of ICAM-1.

Requirement of enzymatic activity for PR3 to induce IL-8 and MCP-1 production and ICAM-1 expression in oral epithelial cells

We then examined whether the PR3-induced activation of oral epithelial cells was due to the enzymatic activity of PR3. It has been reported that the enzymatic activity of PR3 was inhibited by sulfonyl fluoride-type serine proteinase inhibitors such as PMSF and diisopropyl fluorophosphate, and by a naturally occurring serine proteinase inhibitor α1-AT (21). Therefore, we first examined the inhibitory effects of these sulfonyl fluoride-type serine proteinase inhibitors, PMSF and α1-AT on the enzymatic activity of PR3 using Boc-Ala-ONp as a substrate. PR3 showed substantial enzymatic activity (9.7 U/mg protein), and these inhibitors almost completely inhibited it (Fig. 3A). Concurrent with this, TNF-α and α1-AT significantly inhibited...
PR3-induced IL-8 and MCP-1 production, and ICAM-1 expression by HSC-2 cells (Fig. 3, B–D). In addition, FCS almost completely inhibited the enzymatic activity of PR3 (Fig. 3A). PR3-induced IL-8, and MCP-1 production (Fig. 3, B and C) and ICAM-1 expression (Fig. 3D), probably due to naturally occurring protease inhibitors in the serum. By contrast, the serine proteinase inhibitors and serum did not inhibit IL-1β expression (Fig. 3D), probably due to naturally occurring proteinase inhibitors in the serum. The supernatants were collected, and the concentrations of IL-8 (B) and MCP-1 (C and E) were measured by ELISA. The cells were collected by CDS, and the surface expression of ICAM-1 was evaluated by flow cytometry (D). The percentage of cytokine production and ICAM-1 expression was calculated on the basis of the value obtained without inhibitors (B, 2.9 ± 0.2 ng/ml; C; 14.0 ± 1.1 ng/ml; D; mean fluorescence intensity = 465; E, 14.2 ± 0.9 ng/ml). Error bars indicate SD. **, p < 0.01 compared with PR3 alone. The results presented were representative of three different experiments demonstrating similar results.

Analysis of the PAR family in oral epithelial cells and induction of PAR-2 expression on the cell surface by PR3

Because it has been shown that PARs on human platelets and endothelial cells are activated or inactivated by various proteinases (1–3), the expression of the PAR family in oral epithelial cells was analyzed. PBMCs were used as a positive control (5). Oral epithelial cells in culture strongly expressed PAR-1 and PAR-2 mRNA, weakly expressed PAR-3 mRNA, and did not express PAR-4 mRNA, as assessed by RT-PCR analysis (Fig. 4A), which was consistent with the findings of immunohistochemistry using sections of human gingival tissues (9). By flow cytometric analyses, weak expression of PAR-2 was detected on the untreated cell surface, but the expression of PAR-1 and -3 on the cell surface was below the detectable limit (Fig. 4B). The expression of PAR-2 was markedly augmented by PR3 treatment for 1 h, an effect that was inhibited by cytochalasin B, an inhibitor of actin polymerization (39), but not by cycloheximide, a protein synthesis inhibitor. The up-regulation by PR3 was detected at 30 min incubation, and reached a plateau at 1 h (Fig. 4C). Exposure of the cells to PR3 for up to 24 h did not further up-regulate the expression of PAR-2, and did not induce expression of PAR-1 and -3 (data not shown). Furthermore, trypsin, an agonist for PAR-2 (1–3), and a PAR-2 agonist peptide SLIGKV also up-regulated PAR-2 expression (Fig. 4D), but did not up-regulate PAR-1 and -3 (data not shown) on the epithelial cell surface. These results indicate that PAR-2 is an inducible receptor from internal storage by PR3 and known PAR-2 agonists.

Cleavage of human PAR-2 peptide with exposure of its tethered ligand by PR3

The above observation suggests that PR3 cleaves PAR-2 at the specific site and exposes its tethered ligand. To examine this possibility, a peptide corresponding to region surrounding the cleavage site of the human PAR-2 (Fig. 5A) was incubated with PR3, and proteolytic fragments were analyzed. Trypsin, an agonist for PAR-2 (1–3), was used as a positive control. The PAR-2 peptide was rapidly cleaved at the site, R36-S37, by 5 nM PR3 or trypsin (Fig. 5B). The major peptide fragment was identified to be SLIGKVDGT, the PAR-2 tethered ligand, by sequencing. The measured molecular mass (890.8) was in good agreement with the calculated value (890.2). The fragment, SSKGR, was not detected
probably because it was further cleaved to SSK and GR. Upon digestion with 500 nM PR3 or trypsin, the large fragment was cleaved at the site K41-V42, to give another major peak, which corresponded to SLIGK (measured value, 517.9; calculated value, 517.8). PR3 from two different sources showed the identical result. In contrast, thrombin, which does not activate PAR-2 (1–3), did not cleave the PAR-2 peptide.

Involvement of PAR-2 in the PR3-induced activation of oral epithelial cells

To confirm that PR3-induced activation of oral epithelial cells is mediated by PAR-2, Ca2+ mobilization in the cells was measured on exposure of KB cells to PR3, trypsin, and synthetic PAR agonist peptides. Trypsin induced a Ca2+ response, and abolished the response to a second application of trypsin, although the cells responded to the PAR-1 agonist peptide, SFLLRN (Fig. 6A). PR3 as well as the PAR-2 agonist peptide SLIGKV induced the mobilization of Ca2+ in the cells the same as trypsin (Fig. 6B and F). SLIGKV stimulation rendered cells refractory to subsequent stimulation with PR3, but not SFLLRN (Fig. 6C), while stimulation with SFLLRN had no such effect (Fig. 6D). Furthermore, trypsin inhibited a subsequent response to PR3 and to SLIGKV (Fig. 6E), and PR3 also desensitized responses to PAR-2 agonists, trypsin (Fig. 6G), and SLIGKV (Fig. 6H). HSC-2 and PR3 from two different sources showed the same results, although the Ca2+ mobilization in HSC-2 was lower than that in KB (data not shown).

These results clearly indicated that the PR3-induced activation of the cells is mediated by PAR-2.

PR3 induced only marginal response in KB cells at 0.01 μM, and induced a good response at 1 μM, which pattern was the same as that with trypsin, as assessed by Ca2+ mobilization (Fig. 7), indicating that the potency and efficacy are basically the same as those of trypsin. PAR-2 agonist peptide SLIGKV required 100 times greater concentration to induce the same response.

In common with many G protein-coupled receptors, the principal mechanism of PAR-mediated activation is through Gq protein, resulting in activation of PLC (1–3). To examine that PLC is also involved in PR3-induced activation of oral epithelial cells, HSC-2 cells were stimulated with PR3 in the presence of the PLC inhibitor U73122 or the control compound U73343 for 24 h. The PAR-1 and -2 agonist peptides were used as control, and both peptides induced production of IL-8 and MCP-1 (Fig. 8), indicating that the epithelial cells are activated through not only PAR-2, but also PAR-1. The inhibition of PLC completely abolished the production of cytokine induced by both agonist peptides and PR3.

Activation of oral epithelial cells by stimulated neutrophils

To examine whether stimulated neutrophils activate oral epithelial cells, confluent monolayers of HSC-2 were cocultured with indicated number of neutrophils in the presence or absence of FMLP for 24 h. Neutrophils at 0.5 × 10⁷ cells with 0.01 μM FMLP started to induce production of IL-8 from the cells, and increasing
cleaved the PAR-2 peptide with exposure of its tethered ligand; 4) trypsin and a synthetic PAR-2 agonist peptide induced the mobilization of intracellular Ca\(^{2+}\), and rendered cells refractory to subsequent stimulation with PR3; and 5) a PAR-2 agonist peptide as well as PR3 induced cytokine production by the cells, which was inhibited by the PLC inhibitor.

Lactate dehydrogenase activity was not detected in the supernatant of the epithelial cells after PR3 treatment, as previously reported (27), which indicates that PR3 has no adverse effect on the epithelial cell viability. Evidence for the activation of oral epithelial cells by PR3 includes: 1) the expression of IL-8, and MCP-1 and ICAM-1 mRNA was increased and induced by PR3, respectively (Figs. 1 and 2), and 2) PR3 induced the mobilization of intracellular Ca\(^{2+}\) in the epithelial cells (Fig. 6). In contrast to PR3, two other neutrophil serine proteinases, HLE and Cat G, showed only marginal activity on oral epithelial cells, as assessed from the production of MCP-1 (Fig. 1C). HLE and Cat G also induced the mobilization of intracellular Ca\(^{2+}\) in oral epithelial cells to a small extent, compared with PR3 and trypsin, and PR3, trypsin, and a PAR-2 agonist peptide rendered cells refractory to subsequent stimulation with HLE and Cat G (data not shown), although it is not clear whether HLE and Cat G inactivate PAR-1 by cleaving the cell surface tethered ligand domain (10). The findings indicate that PR3 is involved in activating oral epithelial cells through PAR-2 among the three neutrophil serine proteinases.

It is reported that PR3 has a elastase-like specificity for Ala, Ser, and Val amino acids (13), but there was no evidence that it cleaves Arg–Ser or Arg–Val bonds. The present study showed that PR3 rapidly cleaved between Arg and Ser and relatively inefficiently between Lys and Val, which was the same specificity as trypsin and trypsin-like (Fig. 5B) (37), indicating that the R\(^{36}\)-S\(^{37}\) site that surrounds the site of the PAR-2 are structurally accessible to trypsin, tryptase, and PR3, but not by thrombin.

The present study showed that PR3 promoted the surface expression of PAR-2 on oral epithelial cells, which was inhibited by cytochalasin B, but not by cycloheximide (Fig. 4B). The longer incubation with PR3 for up to 24 h neither up-regulated PAR-2 expression nor induced PAR-1 and -3 on the cell surface (data not shown). This indicates that PAR-2 was inducible receptor from intracellular storage, and not required de novo synthesis among the PAR family. In support of this, it was reported that the expression of PAR-2 on human endothelial cells was also up-regulated by LPS as well as IL-1\(\alpha\) and TNF-\(\alpha\) without an effect on PAR-1 expression (40), and that PAR-2 expression in asthmatic bronchial epithelium was significantly increased in comparison with normal epithelium (41). Recently, the PAR-2-induced activation of keratinoctyes was shown to be mediated by the mitogen-activated protein kinase and NF-\(\kappa\)B pathways (42), which are important in the gene expression of cytokines. Thus, it is conceivable that the activation of oral epithelial cells by PR3 is mediated by these pathways involved in producing inflammatory cytokines and expressing adhesion molecules on the cell surface.

PR3 is secreted as a soluble form by activated neutrophils, and also exists as a membrane-bound form on neutrophils. Previous study demonstrated that each azurophil granule of neutrophils contains PR3 at 13.4 mM, and the activation of neutrophils resulted in about a 10-fold increase in membrane-bound PR3 (14), indicating that the local concentration of PR3 around activated neutrophils is high enough to activate PAR2. In addition, although serum contains abundant naturally occurring proteinase inhibitors, membrane-bound PR3 is substantially resistant to inhibition by naturally occurring inhibitors such as \(\alpha\)-AT and elafin, even when these inhibitors are used at a 100- to 300-fold molar excess over the enzyme (14). In support of this possibility, the results in Fig. 9...
showed that activation of oral epithelial cells by FMLP-stimulated neutrophils was only partially inhibited by serine proteinase inhibitors and serum, suggesting that the activation is likely to occur in vivo.

The present study showed that oral epithelial cells are activated to produce IL-8 and MCP-1 and highly express ICAM-1 on the cell surface in response to PR3 through the PAR-2 pathway. IL-8 is a major chemokine responsible for the activation of neutrophils and migration of neutrophils and T cells to inflammatory sites (43). MCP-1 plays a critical role in the activation and migration of monocytes, T cells, and NK cells, and is an important factor in the development of Th1 and Th2 responses (44, 45). ICAM-1 is one of

FIGURE 6. Effect of trypsin, PR3, and PAR agonist peptides on calcium mobilization in oral epithelial cells. A–H, Fura 2-loaded KB cells were exposed to 1 μM trypsin, 10 μg/ml PR3, 100 μM PAR-1 agonist peptide (SFLRN, PAR-1AP), and 100 μM PAR-2 agonist peptide (SLIGKV, PAR-2AP) as indicated sequence, and the change in intracellular calcium was monitored. The results presented were representative of three different experiments demonstrating similar results.

FIGURE 7. Potency and efficacy of PR3 compared with those of other PAR-2 agonists. Fura 2-loaded KB cells were exposed to PR3, trypsin, and PAR-2 agonist peptide (SLIGKV, PAR-2AP) at the concentrations indicated, and the change in intracellular calcium was monitored. PR3, 1 μM = 29 μg/ml; trypsin, 1 μM = 24 μg/ml; PAR-2AP, 100 μM = 61.6 μg/ml. The results presented were representative of three different experiments demonstrating similar results.

FIGURE 8. Effect of PLC inhibition on the PR3-induced production of IL-8 and MCP-1 by oral epithelial cells. Confluent HSC-2 cells were pre-treated with or without U73122 or U73343 at the dose indicated for 30 min. Then cells were stimulated with the PAR-1 agonist peptide (PAR-1AP; 100 μM), the PAR-2 agonist peptide (PAR-2AP; 100 μM), or PR3 (10 μg/ml) for 24 h. Concentrations of IL-8 and MCP-1 in the culture supernatants were determined by ELISA. Error bars indicate SD. **p < 0.01 compared with the respective control (PAR-1AP, PAR-2AP, or PR3 alone). The results presented were representative of three different experiments demonstrating similar results.
the major adhesion molecules interacting with the GCF, including LFA-1 and Mac-1 present on neutrophils, monocytes, and T cells (46). Fig. 2 shows that ICAM-1 expression on oral epithelial cells in response to PR3 contributes extensively to the interaction with neutrophils (via Mac-1 and LFA-1) and that ICAM-1-mediated interaction may further augment the activation of oral epithelial cells through PAR-2. PR3 is another cytokine closely involved in controlling Th1 responses and we recently showed that oral epithelial cells produce massive IL-18 when stimulated by PR3, similar to IFN-γ priming (27). The inflammatory mediator ICAM-1 is characteristic of the infiltration of neutrophils. Infiltration of neutrophils into gingival tissues is an early event in gingival inflammation, and neutrophils are the predominant leukocytes in the gingival crevicular fluid (GCF) (∼90%) (48). It is also evident that GCF has neutrophil serine proteinase activities against McOSuc-Ala-Ala-Pro-Val-p-nitroanilide for HLE and Suc-Ala-Ala-Pro-Val-p-nitroanilide for Cat G (49). Because McOSuc-Ala-Ala-Pro-Val-p-nitroanilide is a PR3 substrate as well (13), PR3 is most likely present in GCF. Therefore, the present study as well as our previous study (27) suggest that the PAR-2-mediated activation of oral epithelial cells by PR3, which is released by active neutrophils, further augments accumulation of neutrophils and immune cells by controlling Th1 or Th2 responses at periodontitis sites, and consequently plays an important role in host defense against periodontal pathogens.

PAR-2 is expressed in the gastrointestinal tract, pancreas, kidney, liver, airway, prostate, ovary, eye, and skin, and is found in epithelial and endothelial cells, smooth muscle cells, keratinocytes, T cell lines, and certain tumor cells (1–3). It was recently reported that stimulation of PAR-2 with an agonist peptide activates human keratinocytes (42), eosinophils (50), and respiratory epithelial cells (51) to induce inflammatory mediators, and up-regulates keratinocyte phagocytosis (52). PR3 is a major target Ag of anti-neutrophil cytoplasmic Abs (15, 16) and degrades extracellular matrix proteins (13). PR3 is also shown to activate many types of cells (12, 17–20), although the underlying mechanism was unclear. Therefore, the present study may provide one of the mechanisms of cell activation by PR3 through PAR-2, and suggests that PAR2 may activate the PAR-2-expressing cells, and regulate a number of inflammatory processes, and that the control of PAR2-activating proteinases including PR3 at inflammatory sites might be beneficial in the regulation of inflammation.

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References


Letter of Retraction


This retraction follows an investigation by Tohoku University into scientific misconduct. The investigation pointed out the following:

1. Fig. 1E: HSC-2, an oral epithelial cell line, is different from KB cells, but the patterns of their band staining for IL-8, MCP-1, and GAPDH cDNA are the same.
2. Fig. 2D: Bands for MCP-1 and GAPDH cDNA in Fig. 1E and ICAM-1 and GAPDH cDNA in this figure are the same.
3. Fig. 4A: Total RNA was extracted from KB (lane 2), HSC-2 (lane 3), and PBMCs (lane 4), and cDNA was prepared and analyzed for the expression of PAR1-4 and GAPDH by RT-PCR. However, three bands of PAR3 and a band of PAR4 are the same. Furthermore, the patterns of GAPDH in this article and those in Fig. 1B and 2A of The Journal of Immunology, 2003, 170: 5690–5696 and in Fig. 2A of Clinical and Diagnostic Laboratory Immunology, 2003, 10: 286–292 are the same.
4. Fig. 4B and 4D: Three panels of the expression of PAR1 with PR3 and PR3 + cytochalasin B (Cyto B) and PAR3 with PR3 + Cyto B are the same. Two panels of the expression of PAR2 with PR3 + cycloheximide (CHX) in Fig. 4B and with trypsin in Fig. 4D are the same.

The first author, who conducted these experiments, could not counter the argument by adducing raw data at the investigation, and the investigation recognized them as scientific misconduct. Therefore, we wish to retract the article.

We deeply regret these errors and apologize to the scientific community for the need to retract the article.

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