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DX-9065a inhibits proinflammatory events induced by gingipains and factor Xa

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Objective: Arginine-specific cysteine proteases (Rgps) from *Porphyromonas gingivalis* are important virulent factors of periodontal diseases. However, there is no therapeutic drug that inhibits proinflammatory events induced by these enzymes. In this study, we investigated proinflammatory activities of Rgps and activated coagulation factor X (FXa) and examined the effect of DX-9065a, a new selective inhibitor of FXa, on proinflammatory events induced by these proteinases.

Methods: Human gingival fibroblasts were stimulated with Rgps and FXa in the presence or absence of DX-9065a, and then interleukin-6 (IL-6) and matrix metalloproteinase-1 (MMP-1) release, their mRNA expression, and nuclear factor κB (NF-κB) activation were assessed using an enzyme-linked immunosorbent assay (ELISA), northern blotting, and a gel-mobility shift method, respectively.

Results: Rgps and FXa activated IL-6 and MMP-1 release in human gingival fibroblasts through their amidolytic activities and in mitogen-activated protein kinase (MAPK) and NF-κB dependent manners. DX-9065a inhibited FXa-induced IL-6 mRNA expression and NF-κB activation. DX-9065a inhibited amidolytic activities of FXa and Rgps *in vitro* and *ex vivo*.

Conclusion: Rgps and FXa are potent inflammatory mediators and DX-9065a may be a useful therapeutic drug for periodontal disease.

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Porphyromonas gingivalis is a Gram-negative anaerobic bacterium that is implicated as an important etiological agent of adult periodontal diseases (1, 2). Proteinases with 'trypsin-like activity', which are bound to membranes of *P. gingivalis* cells, are thought to be important pathogenic agents (3–6). Arginine- and lysine-specific cysteine proteinases (RgpA, RgpB, and Kgp), the major components of the bacterial 'trypsin-like' activities, exhibit various pathogenic activities (7–10). Rgps activate blood coagulation factor X to generate activated factor X (FXa) (11). FXa induces the production of proinflammatory cytokines, such as interleukin-6 (IL-6), IL-8, and macrophage chemotactic protein (12). Therefore, FXa as well as Rgps may induce inflammation in periodontal tissues. However, we have no information on the pathogenic role of FXa in periodontitis.

Some antimicrobial reagents for periodontal disease have been used to eliminate or suppress the growth of putative periodontopathic bacteria (13, 14). However, there are few drugs that can suppress the virulence of *P. gingivalis*, which causes inflammatory responses in periodontal tissue (15, 16). DX-9065a is a developed inhibitor specific for FXa (17). DX-9065a at concentrations higher than those for FXa inhibition can inhibit activities of 'trypsin-like' proteinases, including plasma kallikrein (18), indicating the possibility of this compound as an inhibitor of Rgp and/or Rgp-induced inflammatory events.

To determine the efficacy of DX-9065a as a drug for treatment of periodontal disease, we examined the

proinflammatory activities of Rgps and FXa and the effects of DX-9065a on events induced by these proteinases.

Material and methods

Specimens and probes

DX-9065a was provided by Daiichi Pharmaceutical Co. (Tokyo, Japan). Human factor Xa (FXa) was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN, USA), dansyl-Glu-Gly-Arg (DEGR)-chloromethyl ketone FXa was from Haematologic Technologies (Essex Junction, VT, USA), recombinant human tumor necrosis factor alpha (TNF-α) was from Endogen (Cambridge, MA, USA), leupeptin, chymostatin, E-64, phosphoramidon, Z-Pyr-Gly-Arg-4-methyl-coumaryl-7-amide (Z-Pyr-Gly-Arg-MCA), and Boc-Val-Leu-Lys-MCA were from Peptide Institute, Inc. (Osaka, Japan), and o-phenanthroline was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PD98089, SB203580, and SN50 were from Calbiochem (Cambridge, MA, USA). The selective protease-activated receptor-2 (PAR-2) agonist peptide SLIGRL-NH_2 was from Bachem AG (Bubendorf, Switzerland). A plasmid containing IL-6 cDNA was kindly provided by T. Hirano (Osaka University Medical School, Osaka, Japan), and a plasmid containing human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was kindly provided by I. Sakiyama (Chiba Cancer Center Research Institute and Hospital, Chiba, Japan).

Cells

Human gingival fibroblasts established from explants of healthy gingival tissue were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) in plastic culture dishes with medium change every 3 days for 10–15 days until confluent cell monolayers were formed. After three to four subcultures, homogeneous, slim, spindle-shaped cells (fibroblast-like cells) growing in characteristic swirls were obtained. These primary cultures of human gingival fibroblasts were used as confluent monolayers at subculture passages 5 through 10.

Gingipains

Trypsin-like cysteine proteinases (RgpA, RgpB, and Kgp) were isolated from *P. gingivalis* HG66 culture medium according to the method described by Pike *et al.* (19). The amount of active enzyme in each purified proteinase was determined by active site titration using Phe-Pro-Arg (FPR)-chloromethyl ketone (20).

Measurement of interleukin-6 and matrix metalloproteinase-1 production levels

Human gingival fibroblasts monolayers were preincubated with fetal bovine serum-free DMEM for 24 h. The cells were washed and then stimulated with various concentrations of FXa, Rgps, or SLIGRL-NH_2 in fetal bovine serum-free DMEM. After a further incubation for 1–48 h, the supernatant and sediment were collected and used in subsequent experiments. In some experiments, human gingival fibroblasts were pretreated with the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059 or the p38 inhibitor SB203580 for 10 min and then incubated with 170 nM FXa or 10 nM RgpA for 24 h, and the supernatant and sediment were collected and used in subsequent experiments. In the experiments on inhibition of DX-9065a, human gingival fibroblasts were incubated with 17 nM FXa, 10 nM RgpA, or 10 nM RgpB for 24 h in DMEM in the presence of various concentrations of DX-9065a. The culture supernatants were collected, and then the IL-6 and matrix metalloproteinase-1 (MMP-1) concentrations in each supernatant were measured by using an IL-6 enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Minneapolis, MN, USA) and an MMP-1 ELISA kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), respectively. Human gingival fibroblasts were incubated with 170 nM FXa or 10 ng/ml TNF-α in DMEM in the presence of 17.5 μM DX-9065a. The sediments were collected, and then the expression of IL-6 mRNA and the activation of nuclear factor (NF)-κB in each sediment were examined by northern blot analysis and a gel mobility shift assay, respectively.

Reverse transcription–polymerase chain reaction for protease-activated receptors 1–4

Total RNA was extracted using TRIzol reagent according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized by reverse transcriptase using a commercial reverse transcription–polymerase chain reaction (RT–PCR) kit (Takara Biomedicals, Shiga, Japan), and the reaction was performed following the manufacturer's instructions. Briefly, 1 μg of RNA was added to a 20-μl reaction volume containing 1 μl of random primers, 2 μl of 10 × first-strand buffer, 0.16 μl of RNase inhibitor (1 U/μl), 8 μl of 1 mM dNTP, and 0.14 μl of reverse transcriptase (0.25 U/μl). The reaction mix was incubated at 37°C for 60 min and then at 90°C for 5 min. The resulting cDNA mixture was amplified with *Taq* polymerase, and the following specific primers were synthesized by Hokkaido System Science (Mokkaido, Japan): PAR-1, 5′-TACACCGGAGTGTTTGTAGT-3′ and 5′-TTGAGGACGAGAGGCACTAC-3′ (PCR product, 395 bp; GenBank accession no. M62424); PAR-2, 5′-GGTAAGGTTGATGGCACATC-3′ and 5′-TGGTCTGCTTCACGACATAC-3′ (PCR product, 509 bp; GenBank accession no. U34038); PAR-3, 5′-ATCTCATAGCTTTGTGCCTG-3′ and 5′-CACGCCTGTAATCCAGCACT-3′ (PCR product, 488 bp; GenBank accession no. U92971); PAR-4, 5′-AGTCTGTGCCAATGACAGTG-3′ and 5′-TCATGGCAGAGCACGCGATC-3′ (PCR product, 534 bp; GenBank accession no. AF055917); GAPDH, 5′-CATCACCATCTTCCAGGAGC-3′ and 5′-CATGAGTCCTTCCACGATACC-3′ (PCR product, 286 bp). Amplification conditions were 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for

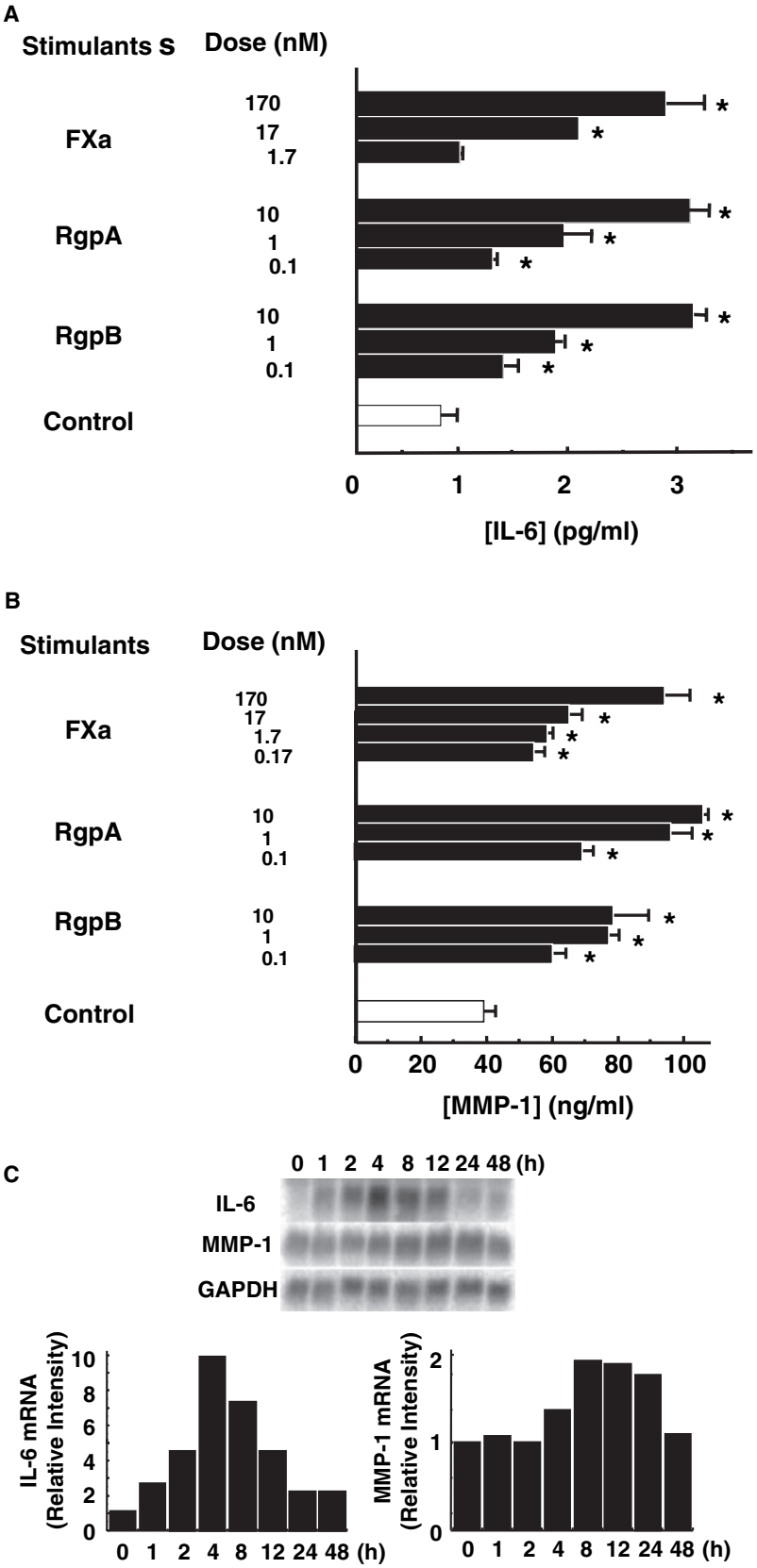

Fig. 1. Activated factor X (FXa) and arginine-specific cysteine proteases (Rgps) activate interleukin-6 (IL-6) and matrix metalloproteinase-1 (MMP-1) induction. (A) FXa and Rgps activate IL-6 production. Increasing amounts of FXa, RgpA, and RgpB were added to human gingival fibroblasts for 24 h, and the amounts of IL-6 released into media were measured by an enzyme-linked immunosorbent assay (ELISA) ($n = 3 \pm$ SD $^*p < 0.01$ vs. control). (B) FXa and Rgps induce MMP-1 production. Increasing amounts of FXa, RgpA, and RgpB were added to human gingival fibroblasts for 24 h, and the amounts of MMP-1 released into media were measured by an ELISA ($n = 3 \pm$ SD $^*p < 0.01$ vs. control). (C) FXa enhances IL-6 and MMP-1 mRNA expression. Human gingival fibroblasts were stimulated with 170 nM FXa for 0–48 h and the mRNA expressions of IL-6 and MMP-1 in the cells were analyzed by northern blotting. The intensity of IL-6 and MMP-1 signals was expressed as fold increase over control (0 h). (This experiment was repeated twice with similar results.)

90 s for PAR-1 and PAR-2; 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 90 s for PAR-3 and PAR-4; and 28 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 90 s for GAPDH.

Northern blot analysis

Total RNA from human gingival fibroblasts was resolved by electrophoresis through a 1.2% agarose gel, transferred to a nylon membrane (Zeta-Probe, Bio-Rad Laboratories, Richmond, CA, USA), and hybridized with ^{32}P-labeled IL-6 cDNA probes. The results are expressed as relative mRNA accumulation, using GAPDH mRNA as an internal standard.

Gel mobility shift assay

Nuclear protein extracts were incubated with synthetic oligonucleotide probes for wild-type NF-κB (5′-AGCTTGGGGACTTTCCGAG-3′) labeled by [γ-^{32}P]ATP for 30 min, loaded onto a 5% polyacrylamide gel, and then electrophoresed. The gels were dried and autoradiographed by exposure to X-ray film at −80°C.

DX-9065a inhibition of amidolytic activity

Various concentrations of DX-9065a were added to each proteinase (1 μM) in 0.1 M Tris-HCl, pH 7.6, containing

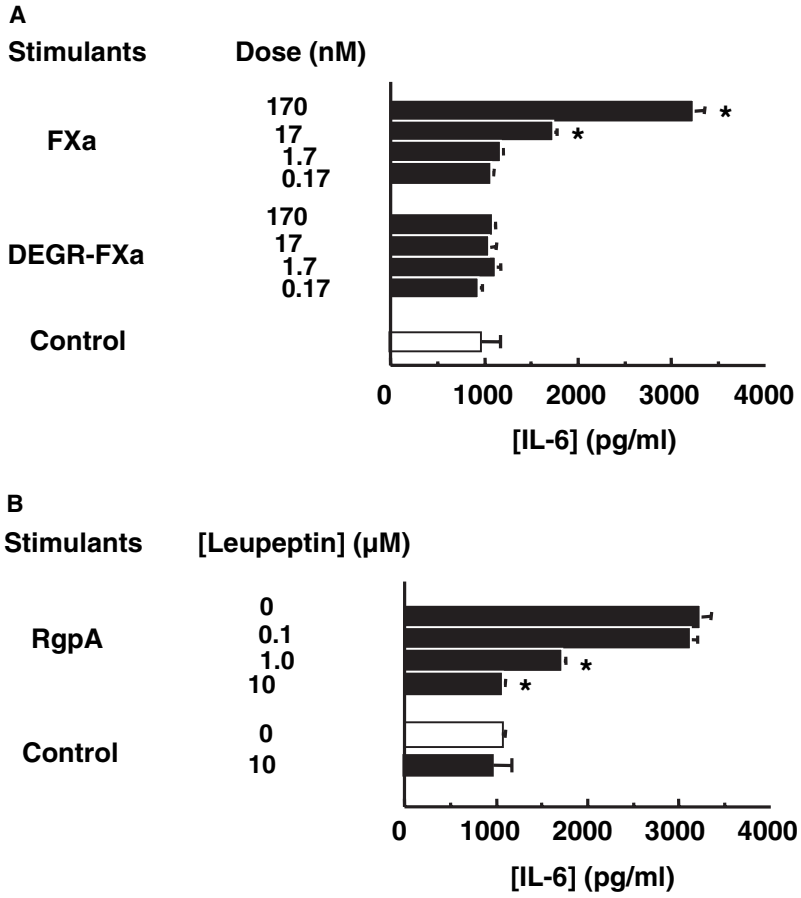

Fig. 2. Activated factor X (FXa) and arginine-specific cysteine protease (Rgp) activate interleukin-6 (IL-6) induction through their amidolytic activities. (A) DEGR-FXa does not induce IL-6 production. Increasing amounts of FXa and DEGR-FXa were added to human gingival fibroblasts for 24 h, and the amounts of IL-6 released into media were measured by an enzyme-linked immunosorbent assay (ELISA). ($n = 3 \pm$ SD $^*p < 0.01$ vs. control). (B) Leupeptin blocks RgpA-induced IL-6 production. Human gingival fibroblasts were incubated for 24 h with 10 nM RgpA pretreated with leupeptin. The amounts of IL-6 released from cells into the media were measured by an ELISA. ($n = 3 \pm$ SD $^*p < 0.01$ vs. RgpA alone).

150 mM NaCl, 0.1 mM cysteine and 0.1 mg/ml BSA. Then a substrate (10 mM) (Z-Pyr-Gly-Arg-MCA for Rgps and FXa, and Boc-Val-Leu-Lys-MCA for Kgp) was added to the mixture. The amount of 7-amino-4-methyl coumarin (AMC) released by a proteinase at 37°C was measured by a fluorimeter (Model 650–40, Hitachi).

Trypsin-like activity in gingival crevicular fluids

Gingival crevicular fluid samples were collected from eight adult periodontitis patients (four men and four women; mean age, 54 years; range, 34–66 years) according to standard methods on filter paper strips and stored at −80°C. Proteinase activity was measured by incubating with 50 μM Z-Pyr-Gly-Arg-MCA at 37°C for 30 min, and MCA liberation was measured by fluorescence intensity (Ex. 355 nm, Em. 460 nm) using a Multilabel counter (ARVO 1420; Amersham Pharmacia Biotech). In some experiments, 100 μM DX-9065a was simultaneously added to the samples, and then fluorescence intensity was measured.

Statistical analysis

The significance of differences between each test specimen and the respective control was determined by Student's *t*-test.

Results

Arginine-specific cysteine proteases and factor Xa activate interleukin-6 and matrix metalloproteinase-1 induction *in vitro*

To determine whether Rgps and FXa stimulate induction of proinflammatory mediators, we examined the effects of FXa and Rgps on induction of IL-6 and MMP-1 in human gingival fibroblasts. We stimulated human gingival fibroblasts with RgpA, RgpB and FXa for 16 h and then measured the amounts of IL-6 and MMP-1 released in the media. Rgps and FXa augmented IL-6 production (Fig. 1A). The level of IL-6 in the culture supernatant of human gingival fibroblasts was elevated at 4 h after stimulation with 170 nM FXa or 10 nM RgpA and continued to increase at least up to 48 h (data not shown). Rgps and FXa also induced production of MMP-1 by human gingival fibroblasts (Fig. 1B). FXa strongly induced IL-6 mRNA expression and also enhanced MMP-1 mRNA expression in human gingival fibroblasts (Fig. 1C). IL-6 mRNA expression by human gingival fibroblasts began to increase after 1 h of stimulation with FXa and reached a maximum level after 4 h of stimulation and had decreased by 10% of the maximum level by 24 h. MMP-1 mRNA expression peaked between 8 and 12 h and was maintained at that level until 24 h. To determine whether the enzymatic activity of FXa is necessary for the observed proinflammatory responses, we inactivated FXa by DEGR-chloromethyl ketone. DEGR-FXa failed to induce IL-6 production (Fig. 2A). DEGR-FXa did not induce MMP-1 production (data not shown). Next, we examined the effect of leupeptin, which inactivates amidolytic activity of Rgp, on Rgp-induced IL-6 and MMP-1 production. Leupeptin at 10 μM completely inhibited IL-6 production stimulated by RgpA (Fig. 2B).

Leupeptin–treated RgpA could not activate MMP-1 production (data not shown). These results suggest that Rgps and FXa caused IL-6 and MMP-1 induction through those enzymatic activities.

Mitogen-activated protein kinase and nuclear factor-κB pathways mediate induction of interleukin-6 production by factor Xa and arginine-specific cysteine proteases

To examine the mechanism by which FXa and Rgps activate IL-6 and MMP-1 induction, we first investigated whether the effects of FXa and Rgps on IL-6 and MMP-1 production were mediated by proteolytic cleavage of PAR-2. We examined the expression of PAR-1, 2, 3, and 4 in human gingival fibroblasts by RT–PCR. Human gingival fibroblasts expressed PAR-1, 2, and 3 mRNAs but not PAR-4 mRNA (Fig. 3A). We next examined the production of IL-6 and MMP-1 by human gingival fibroblasts in response to various concentrations of SLIGRL-NH_2, a PAR-2 agonist. SLIGRL-NH_2 caused dose-dependent increases in IL-6 and MMP-1 production levels in human gingival fibroblasts (Fig. 3B). To examine the signal transduction of FXa and Rgps in human gingival fibroblasts with regard to IL-6 induction, we examined the effects of several inhibitors against molecules involved in the mitogen-activated protein kinase (MAPK)-dependent signal transduction pathway of human gingival fibroblasts. PD98089 (an MEK inhibitor) significantly inhibited IL-6 production induced by FXa or RgpA (Fig. 4A). On the other hand, SB203580 (a p38 inhibitor) had no effect on IL-6 production induced by FXa or RgpA. Next, we demonstrated that SN50 (an NF-κB inhibitor) significantly reduced IL-6 production stimulated by FXa or RgpA (Fig. 4B). Furthermore, PD98089 significantly suppressed IL-6 production induced by SLIGRL-NH_2, whereas SB203580 had no effect (data not shown). In addition, SN50 significantly reduced IL-6 production in response to SLIGRL-NH_2 (data not shown).

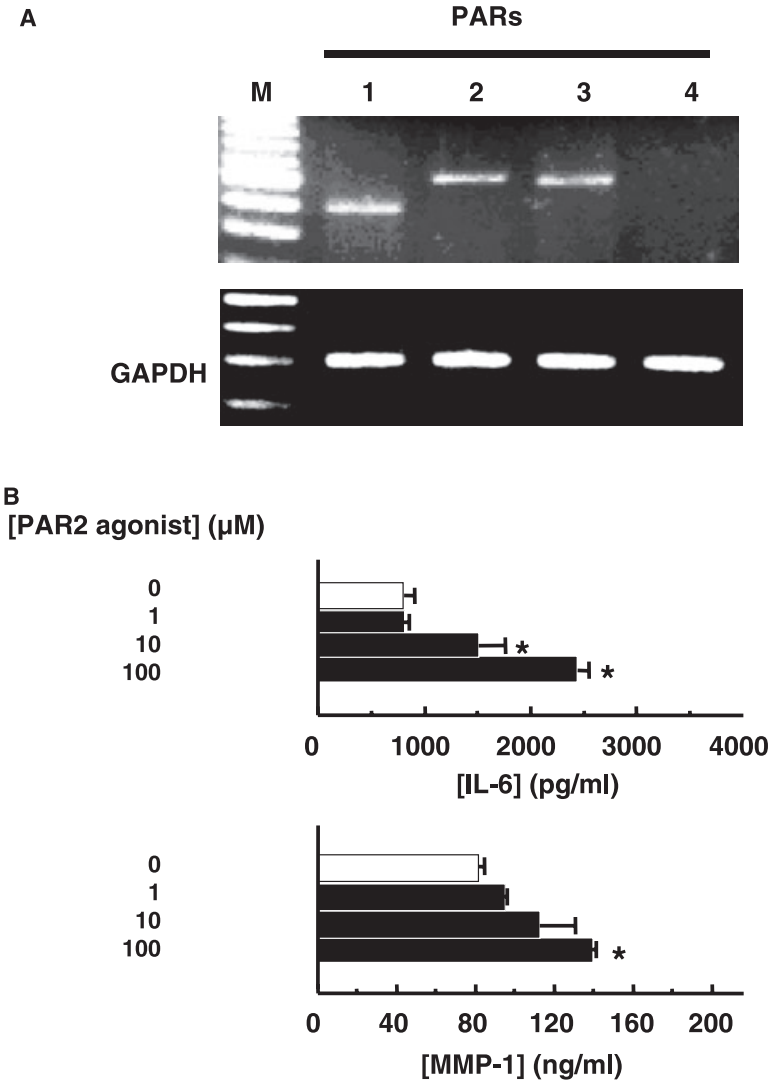

Fig. 3. Protease-activated receptors (PARs) mediate interleukin-6 (IL-6) and matrix metalloproteinase-1 (MMP-1) production. (A) Human gingival fibroblasts express PAR1, 2, and 3 mRNAs. Total RNA was extracted from human gingival fibroblasts and analyzed by reverse transcription–polymerase chain reaction (RT–PCR) for PARs (PAR1, 2, 3, and 4) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This experiment was repeated twice with similar results. (B) PAR2 agonists activate IL-6 and MMP-1 production. Increasing amounts of PAR1 and PAR2 agonists were added to human gingival fibroblasts for 24 h, and the amount of IL-6 and MMP-1 released into media was measured by an enzyme-linked immunosorbent assay (ELISA) ($n = 3 \pm$ SD $^*p < 0.01$ vs. 0 μM).

DX-9065a inhibits factor Xa- and arginine-specific cysteine protease-activated proinflammatory responses

We next examined the effect of DX-9065a on Rgp- and FXa-induced IL-6 production. We stimulated human gingival fibroblasts with 10 nM Rgps and 17 nM FXa in the presence or absence of DX-9065a for 24 h. DX-9065a inhibited Rgp- and FXa-induced IL-6 production in a dose-dependent manner (Fig. 5A). The 50% inhibitory concentrations (IC_{50}) of DX-9065a for FXa, RgpA and RgpB were 0.013 μM, 1.54 μM and 0.28 μM, respectively. DX-9065a also inhibited production of MMP-1 by human gingival fibroblasts (Fig. 5B). The IC_{50} values of DX-9065a for FXa, RgpA and RgpB were 0.028 μM, 1.51 μM and

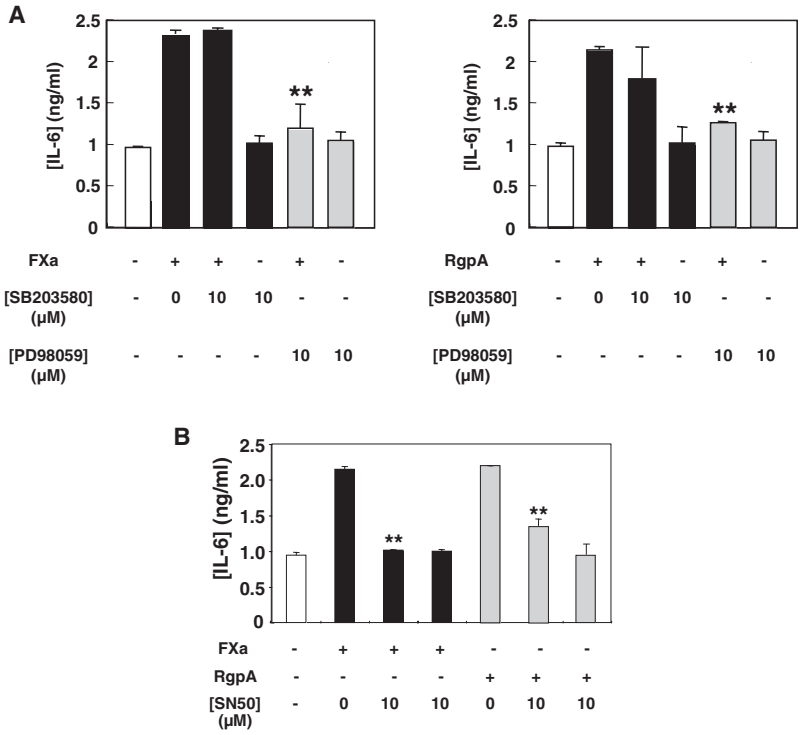

Fig. 4. Mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways mediate activated factor X (FXa) and arginine-specific cysteine protease (Rgp)-activated interleukin-6 (IL-6) production. (A) FXa and RgpA induce IL-6 production through a mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)-dependent pathway. Human gingival fibroblasts were pretreated with the MEK inhibitor PD98059 or the p38 inhibitor SB203580 for 10 min and then incubated with 170 nM FXa or 10 nM RgpA for 24 h. The amount of IL-6 released from cells into the media was measured by an enzyme-linked immunosorbent assay (ELISA) ($n = 3 \pm$ SD $^{**}p < 0.01$ vs. FXa or RgpA). (B) FXa and RgpA induce IL-6 production through a NF-κB-dependent pathway. Human gingival fibroblasts were pretreated with the NF-κB inhibitor SN50 for 30 min and then incubated with 170 nM FXa or 10 nM RgpA for 24 h. The amount of IL-6 released from cells into the media was measured by an ELISA ($n = 3 \pm$ SD $^{**}p < 0.01$ vs. FXa or RgpA).

0.35 μM, respectively. We also examined the effect of DX-9065a on FXa-induced IL-6 mRNA expression. FXa at 170 nM as well as 10 ng/ml TNF-α strongly induced IL-6 mRNA expression in human gingival fibroblasts, and 17.5 μM of DX-9065a inhibited the IL-6 mRNA expression induced by FXa but not that induced by TNF-α (Fig. 5C). Activation of nuclear factor NF-κB has been shown to be crucial for IL-6 transcription (21). Therefore, we next examined the effect of DX-9065a on FXa-induced NF-κB activation. NF-κB activation was clearly observed in human gingival fibroblasts stimulated with 170 μM FXa for 4 h as well as in those cells stimulated with TNF-α (Fig. 5D). DX-9065a strongly inhibited FXa-induced NF-κB activation, whereas the drug did not inhibit TNF-α-induced NF-κB activation (Fig. 5D).

DX-9065a inhibits amidolytic activities of arginine-specific cysteine proteases and factor Xa

To investigate the direct effect of DX-9065a on Rgps, the amidolytic activity of Rgps on Z-Pyr-Gly-Arg-MCA was measured in the presence of DX-9065a. DX-9065a strongly inhibited the amidolytic activity of FXa (Fig. 6A). DX-9065a also inhibited the amidolytic activity of Rgps in a dose-dependent manner at concentrations starting at 10^{-5} M. In contrast, DX-9065a did not inhibit Kgp amidolytic activity.

To determine whether DX-9065a also has an effect on Rgps in periodontal pockets, gingival crevicular fluid samples were collected from patients with adult periodontitis, and the effect of DX-9065a on trypsin-like activity in the gingival crevicular fluid was examined. We first measured the trypsin-like proteinase activity in gingival crevicular fluid samples from patients with adult periodontitis and then examined the effect of DX-9065a on the activity. The level of trypsin-like proteinase activity was significantly higher in gingival crevicular fluid samples from diseased sites than in those from normal sites (Fig. 6B). Furthermore, 100 μM DX-9065a inhibited the activity in gingival crevicular fluid samples from diseased sites.

Discussion

The major finding of this study is that DX-9065a inhibits Rgps and FXa induced inflammatory events *in vitro* and *ex vivo* by blocking amidolytic activities of these proteinases. DX-9065a may be a useful therapeutic drug for periodontal disease.

Arginine-specific cysteine proteases and factor Xa induce inflammation in periodontal tissue

Porphyromonas gingivalis proteolytic enzymes, especially 'trypsin-like' proteinases, are closely associated with the progress of periodontitis (3–6). Two types of cysteine proteases responsible for the so-called trypsin-like activity of the bacterium have been purified (22–24): a lysine-specific protease, termed lys-gingipain (Kgp), and an arginine-specific protease referred to as arg-gingipain. The latter is present as three variants: 50-kDa RgpB, 50-kDa RgpA, and 95-kDa HRgpA. Rgps enhanced vascular permeability through prekallikrein activation or direct bradykinin release, which is potentially associated with gingival edema and crevicular fluid production (7). Rgps degradate fibrinogen/fibrin in human plasma, being involved in the

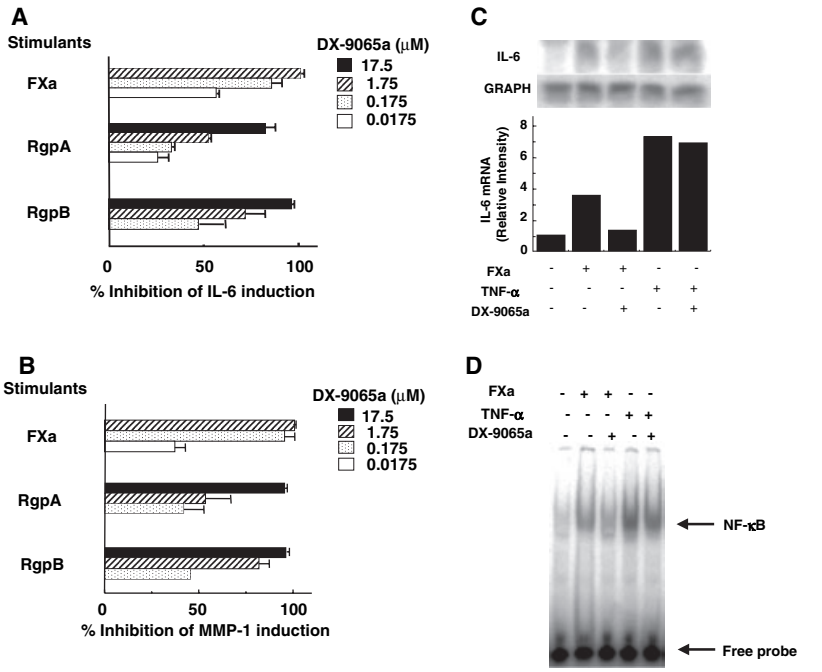

Fig. 5. DX-9065a inhibits activated factor X (FXa) and arginine-specific cysteine protease (Rgp)-evoked proinflammatory responses. (A) DX-9065a inhibits FXa- and Rgp-evoked interleukin-6 (IL-6) production. Human gingival fibroblasts were incubated with 17 nM FXa, 10 nM RgpA, and 10 nM RgpB in the presence or absence of DX-9065a for 24 h. The amounts of IL-6 released into media were measured by an enzyme-linked immunosorbent assay (ELISA) ($n = 3 \pm$ SD). (B) DX-9065a inhibits FXa- and Rgp-evoked matrix metalloproteinase-1 (MMP-1) production. Human gingival fibroblasts were incubated with 17 nM FXa, 10 nM RgpA, and 10 nM RgpB in the presence or absence of DX-9065a for 24 h. The amounts of MMP-1 released into media were measured by an ELISA ($n = 3 \pm$ SD). (C) DX-9065a inhibits FXa-induced IL-6 mRNA expression. Human gingival fibroblasts was incubated with 170 nM FXa or 1 nM tumor necrosis factor alpha (TNF-α) for 4 h in the presence or absence of 17.5 μM DX-9065a. The mRNA expression of IL-6 in the cells was analyzed by northern blotting. The intensity of IL-6 signals was expressed as fold increase over control (unstimulated cells). (This experiment was repeated twice with similar results.) (D) DX-9065a inhibits FXa-induced nuclear factor κB (NF-κB) activation. Human gingival fibroblasts was incubated with 170 nM FXa or 1 nM TNF-α for 4 h in the presence or absence of 17.5 μM DX-9065a. The activation of NF-κB in cells was analyzed by a gel mobility shift assay. (This experiment was repeated twice with similar results.)

bleeding tendency at the diseased gingiva (25). Rgps activate secreted latent MMPs that can destroy periodontal tissues (26). Rgps induces various proinflammatory cytokines, such as IL-8, that evoke neutrophil accumulation at periodontitis sites (27). These findings suggest that Rgps are potent virulence factors of *P. gingivalis* and are likely to be associated with the development of periodontitis.

Rgps also activate FX and prothrombin for blood coagulation (11, 28). Factor Xa activates protease-activated receptor-2 (PAR-2) (29). Proteolytic cleavage of PAR-2 activates leukocytes and mast cells and evokes a proinflammatory response (30–32). Therefore, factor Xa may contribute to the progression of periodontitis. However, the pathological role of FXa in periodontitis has not been clarified. In this study, we showed that FXa strongly induced IL-6 and MMP-1 production in human gingival fibroblasts. In addition, the level of FXa activity was significantly higher in gingival crevicular fluid from diseased sites than in that from normal sites. These results suggest that FXa may lead to progression of inflammation in periodontal tissues.

How do arginine-specific cysteine proteases and factor Xa evoke proinflammatory events?

Rgps and FXa activate PAR-2 (29, 33). We found that human gingival fibroblasts express PAR-1, 2, and 3 but not PAR-4 and that a PAR-2 agonist induces IL-6 and MMP-1 production. Both Rgps and FXa are known to exert their effects through a variety of downstream signaling mechanisms that may involve MEK, p38, PKC and NF-κB (34–36). In this study, we demonstrated the signal transduction of Rgps and FXa within the pathways of MAP kinases and NF-κB. In brief, PD98089, a specific inhibitor of MEK1/2 that blocks the p44/p42 MAPK pathway and NF-κB, suppressed the effects of both RgpA and FXa on IL-6 production. In contrast, SB203580, an inhibitor of p38 MAP kinase, had no effect. Furthermore, PD98089 and SN50 significantly suppressed IL-6 production induced by a PAR-2 agonist, whereas SB203580 had no effect. These facts prove that Rgps and FXa activate PAR-2, p44/p42 MAPK and NF-κB, leading to IL-6 expression.

DX-9065a inhibits arginine-specific cysteine protease- and factor Xa-induced inflammation

Rgps are involved in the progression of periodontitis (3–6). Rgps are important for the bacterium both to exhibit its virulence and to survive in periodontal pockets. Therefore, Rgp inhibitors may be useful for new therapeutic approaches to periodontal diseases. Nevertheless, no proteinase inhibitor has been used as a therapeutic drug for periodontitis. In this study, we showed that DX-9065a inhibited the amidolytic activity of Rgps. The K_i values of DX-9065a for RgpA and RgpB were 14.5 and 13 μM, respectively. DX-9065a strongly inhibited production of IL-6 and MMP-1 from human gingival fibroblasts at a concentration of 17.5 μM, suggesting that this compound is also effective for suppression of Rgp-induced proinflammatory reactions. In addition, a lower concentration of DX-9065a (less than 1 mM) was not toxic to human gingival fibroblasts

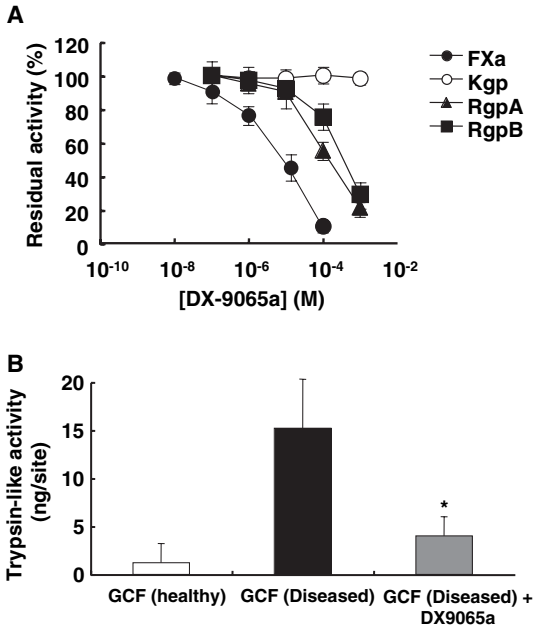

Fig. 6. DX-9065a inhibits proteolytic activities of activated factor X (FXa) and arginine-specific cysteine proteases (Rgps). (A) *In vitro* experiment: 1 μM each of FXa, Kgp, RgpA, and RgpB were, respectively, incubated with various concentrations (10^{-3} to 10^{-9} M) of DX-9065a in the presence of 7-amino-4-methyl coumarin (AMC) at 37°C for 30 min, and the AMC liberation was measured ($n = 3 \pm$ SD). (B) *Ex vivo* experiment: gingival crevicular fluids were collected from eight healthy sites and eight diseased sites from eight patients with adult periodontitis and mixed with or not mixed with 100 μM DX-9065a, and then the trypsin-like activity in each sample was measured by 4-methyl-coumaryl-7-amide (MCA) liberation from the substrate [$n = 8 \pm$ SD $^*p < 0.01$ vs. GCF (Diseased)]. GCF, gingival crevicular fluid.

(LD_{50} was 12.4 mM). Taken together, the results indicate that DX-9065a is a possible therapeutic drug for periodontitis.

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