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Selective inhibition of *Porphyromonas gingivalis* growth by a factor Xa inhibitor, DX-9065a

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Background: Porphyromonas gingivalis is a causative bacterium of adult periodontitis. However, there is no drug specific for *P. gingivalis* and for its virulence factor.

Objectives: The objective of this study was to examine the effects of a new selective inhibitor of activated factor X, DX-9065a, on growth of *Porphyromonas gingivalis* and other periodontopathic bacteria.

Methods: We incubated *P. gingivalis* and other periodontopathic bacteria in the presence or absence of DX-9065a and examined the effect of DX-9065a on bacterial growth and trypsin-like activity in its cultures. We also examined the effects of DX9065a on amidolytic activity of purified trypsin-like proteinases (gingipains RgpA and RgpB), from *P. gingivalis* and on trypsin-like activity in gingival crevicular fluids from patients with adult periodontitis.

Results: DX-9065a selectively inhibited the growth of *P. gingivalis* and *Prevotella intermedia*, and its effect on *P. gingivalis* was bactericidal. Trypsin-like proteinase activity was detected in *P. gingivalis,* and the activity was strongly inhibited by DX-9065a. DX-9065a even inhibited amidolytic activity of RgpA and RgpB from *P. gingivalis*. Furthermore, trypsin-like proteinase activity in gingival crevicular fluids was strongly inhibited by DX-9065a.

Conclusions: DX-9065a inhibits *P. gingivalis* growth in part through to its ability to inhibit the trypsin-like proteinase activity in *P. gingivalis* and may be useful for a new drug for treatment of adult periodontitis.

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Porphyromonas gingivalis is a major cause of adult periodontitis (1). *P. gingivalis* is dependent on amino acids and peptides for its growth (2). The bacterial arginine-specific serine protease Arg-gingipain (Rgp) and the lysine-specific cysteine protease Lys-gingipain (Kgp) are responsible for the degradation of environmental proteins and the generation of amino acids and peptides as a source of energy for the bacteria (3). Therefore, protease inhibitors specific for gingipains may inhibit the growth of *P. gingivalis*. However, little has been discovered of such a drug (4, 5). DX-9065a, a selective factor Xa (FXa) inhibitor, has been shown to strongly and directly inhibit the protease activity of FXa in an anti-thrombin III-independent manner (6). This compound has also been shown to extensively inhibit activities of other proteases associated with blood coagulation and inflammatory responses at a high concentration (7). In this study, we investigated the effects of DX-9065a on the growth of *P. gingivalis* through inhibition of Rgp activity.

Material and methods

Material

DX-9065a was provided by Daiichi Pharmaceutical Co. (Tokyo, Japan). Leupeptin, chymostatin, E-64, phosphoramidon, Z-Pyr-Gly-Arg-MCA, and leupeptin were purchased from Peptide Institute, Inc. (Osaka, Japan). *o*-Phenanthroline was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human Factor Xa (FXa) was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN, USA).

Bacterial strains and growth

We used eight strains of oral bacteria, *P. gingivalis* (381, 6/26, HW24D1, and HG564), *Prevotella intermedia* (ATCC 25611 and ATCC 15033), *Fusobacterium nucleatum* ATCC 10953, and *Actinobacillus actinomycetemcomitans* Y4 (1). As reference bacteria, we used *Actinomyces neaslundii* (ATCC 15987), *Streptococcus mutans* (MT8148), and *Streptococcus sanguinis* (ATCC 10556). The bacteria were cultured as described previously (8).

DX-9065a inhibition of bacterial growth

To determine the effect of DX-9065a on bacterial growth, bacterial cultures were incubated for 24 h and then the cultures were diluted to a final cell density of 5×10^7 colony-forming units (CFU)/ml with Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing various concentrations of DX-9065a or leupeptin and cultured anaerobically at 37°C for 24 h. Growth was monitored by measuring the OD at 660 nm. To examine the bactericidal effect of DX-9065a on *P. gingivalis*, *P. gingivalis* 381 (5×10^8 CFU/ml) was cultured with 700 μM of DX-9065a for 0, 20, 40, and 60 min. After centrifugation of the cultures, the supernatants and sediments were collected. Then RNA contents in the supernatants were measured. The sediments were plated on blood agar plates and were cultured for 7 days, and then the CFU were counted. To clarify the mechanism by which DX-9065a inhibits *P. gingivalis* growth, cultures of *P. gingivalis* incubated for 24 h were diluted to a final cell density of 5×10^7 CFU/ml with α-ketoglutarate/bovine serum albumin (α-KG/BSA) medium containing various concentrations of DX-9065a and cultured anaerobically at 37°C for 48 h. The degree of inhibition of bacterial growth was expressed as percentage inhibition, where % inhibition = [OD at 660 nm in the control cultures (bacteria treated with broth alone) − OD at 660 nm in the test cultures (bacteria treated with DX-9065a or leupeptin)]/(OD at 660 nm in the control cultures) × 100.

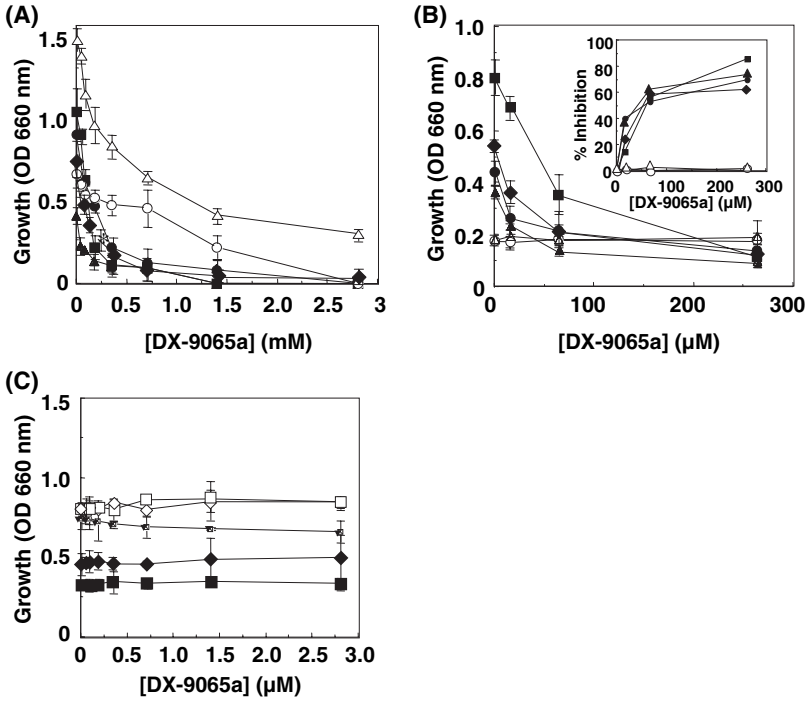

Fig. 1. Effects of DX-9065a on the growth of periodontopathic bacteria. *Porphyromonas gingivalis* and *Prevotella intermedia* (5×10^7 CFU/ml, respectively) were incubated with (A) Gifu anaerobic medium broth or (B) α-ketoglutarate/bovine serum albumin medium containing various concentrations of DX-9065a and cultured anaerobically at 37°C for 24 h. Bacterial growth was monitored by measuring the optical density at 660 nm ($n = 3 \pm$ SD). ●, *P. gingivalis* 381; ▲ , *P. gingivalis* 6/26; ◆ , *P. gingivalis* HG564; ■, *P. gingivalis* HW24D1; ○, *P. intermedia* ATCC 25611, △ , *P. intermedia* ATCC 15033. (C) *Actinobacillus actinomycetemcomitans* Y4 (■), *Fusobacterium nucleatum* ATCC 10953 (◆), *Actinomyces naeslundii* ATCC 10556 (▼), *Streptococcus mutans* MT8148 (□) and *Stretococcus sanguinis* ATCC 10556 (◇) (5×10^7 CFU/ml, respectively) were incubated with various concentrations of DX-9065a and cultured anaerobically at 37°C for 24 h. Bacterial growth was monitored by measuring the optical density at 660 nm ($n = 3 \pm$ SD).

DX-9065a inhibition of trypsin-like activity in bacterial cell cultures

Trypsin-like proteinase activity in bacterial cultures was determined using a synthetic 4-methyl-coumaryl-7-amide (MCA) substrate. Bacterial cultures incubated for 24 h were separated into supernatants and cell pellets. Bacterial pellets were sonicated and mixed with an inhibitor cocktail (0.1 M Tris-HCl, 100 m M NaCl, 2 μM chostatin, 20 μM E-64, 20 μM phosphoramidon, 2 mM *o*-phenanthroline, pH 8.0) with 50 μM Z-Pyr-Gly-Arg-MCA and incubated at 37°C for 30 min. Liberation of MCA was measured by a fluorimeter (Ex. 355 nm, Em. 460 nm) using a Multilabel counter (ARVO 1420, Amersham Pharmacia Biotech, Buckinghamshire,

UK). As a standard enzyme, FXa was used, and the amidolytic activity of the specimens was indicated as an FXa equivalent. In some experiments, various concentrations of DX-9065a were simultaneously added to the mixtures of specimens and substrates, and the effects of the drugs on amidolytic activity were examined.

DX-9065a inhibition of amidolytic activity of gingipains

RgpB and RgpA were isolated from *P. gingivalis* HG66 culture medium (9). Proteinase activity was monitored by incubation with a fluorimeter using Z-Pyr-Gly-Arg-MCA for Rgps and Boc-Val-Leu-Lys-MCA for Kgp. K_i values were determined by a Dixon plot.

Trypsin-like activity in gingival crevicular fluids

Gingival crevicular fluid samples were collected from six adult periodontitis patients (three men and three women; mean age, 51 years; range of ages, 37–64 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, various concentrations of DX-9065a were simultaneously added to the samples and then fluorescence intensity was measured.

Statistical analysis

The 50% inhibitory concentrations (IC_{50}) corresponding to a 50% decrease in OD at 660 nm in drug-free bacterial cultures were determined by non-linear regression analysis of log-dose/response curves. Data were expressed as the geometric mean IC_{50}, and 95% confidence intervals (95% CI) were calculated. Student's *t*-test was used to compare IC_{50} values. The significance of differences between each test specimen and the respective control was determined by Student's *t*-test.

Results

DX-9065a inhibits *Porphyromonas gingivalis* and *Prevotella intermedia* growth

DX-9065a inhibited the growth of four strains of *P. gingivalis* in a dose-dependent manner in GAM broth (Fig. 1A). The IC_{50} of DX-9065a for the bacteria *P. gingivalis* 381, 6/26, HG564 and HW24D1 were 143 μM (95% CI, 128–158 μM), 109 μM (95% CI, 99–121 μM), 96 μM (95% CI, 84–106 μM) and 88 μM (95% CI, 80–96 μM), respectively. The growth of two strains of *P. intermedia* (ATCC 25611 and ATCC 15033) was also inhibited by DX-9065a, although the IC_{50} values of the drug against *P. intermedia* were generally higher [340 μM (95% CI, 328–352 μM) and 513 μM (95% CI, 481–545 μM)]. In contrast, DX-9065a did not inhibit the replication of *A. actinomycetemcomitans*, *F. nucleatum*, *A. naeslundii*, *S. mutans* MT8148 and *S. sanguinis* ATCC 10556 (Fig. 1C). As GAM broth supplies peptides and carbohydrates to the bacteria, the bacteria can use the peptides without processing by bacterial trypsin-like protease. These data show that DX-9065a inhibits bacterial growth by unknown mechanisms, possibly through direct or indirect limitation of bacterial nutrients.

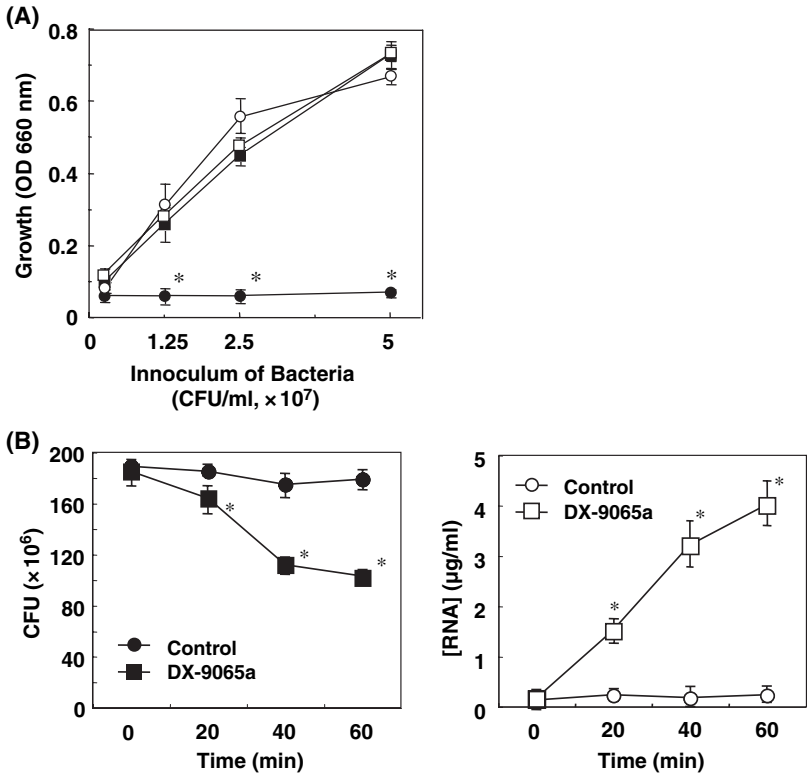

Fig. 2. Bactericidal effect of DX-9065a on *Porphyromonas gingivalis*. (A) *P. gingivalis* 381 and *Prevotella intermedia* ATCC 25611 were incubated with 700 μM of DX-9065a for 24 h and then diluted with Gifu anaerobic medium broth and incubated anaerobically at 37°C for 24 h. Growth was monitored by the optical density at 660 nm ($n = 3 \pm$ SD $^*p < 0.01$ vs. bacteria alone). ●, *P. gingivalis* + DX-9065a; ○, *P. gingivalis*; ■, *P. intermedia* + DX-9065a; □, *P. intermedia*. (B) *P. gingivalis* 381 (5×10^8 CFU/ml) was cultured with 700 μM of DX-9065a for 0, 20, 40, and 60 min. After centrifugation of the cultures, the supernatants and sediments were collected. The sediments were plated on blood agar plates and were cultured for 7 days, and then the colony-forming units (CFU) were counted ($n = 3 \pm$ SD, $^*p < 0.01$ vs. control) (left). RNA contents in the supernatants were measured ($n = 3 \pm$ SD, $^*p < 0.01$ vs. control) (right).

To determine whether DX-9065a inhibits *P. gingivalis* growth via inhibition of Rgp activity, we examined the effects of DX-9065a on the growth of *P. gingivalis* in α-KG/BSA medium. DX-9065a inhibited the growth of *P. gingivalis* 381, 6/26, HG564 and HW24D1 (Fig. 1B). The IC_{50} values of DX-9065a for strains 381, 6/26, HG564 and HW24D1 in α-KG/BSA medium were 58 μM (95% CI, 51–65 μM), 43 μM (95% CI, 38–48 μM), 48.0 μM (95% CI, 39–57 μM) and 58.0 μM (95% CI, 50–66 μM), respectively. The IC_{50} values of DX-9065a were lower for bacteria grown in α-KG/BSA broth than for bacteria grown in GAM broth (*P. gingivalis* 381, $p = 0.021$; *P. gingivalis* 6/26, $p = 0.003$; *P. gingivalis* HG564, $p = 0.006$; *P. gingivalis* 24D1, $p = 0.01$), because bacteria depend on their trypsin-like proteases to process BSA, the only source of protein in this α-KG/BSA broth. DX-9065a did not suppress the growth of *P. intermedia* ATCC 25611 and *P. intermedia* ATCC 15033. Because α-KG/BSA medium supplies proteins but not peptides to bacteria (10), the bacteria must use bacterial trypsin-like proteases to process the proteins in the broth in order to grow. These results suggest that DX-9065a inhibits bacteria growth, possibly by blocking bacterial use of proteins as a source of energy.

To determine whether DX-9065a is bactericidal against *P. gingivalis* and *P. intermedia*, cultures of *P. gingivalis* 381 and *P. intermedia* ATCC 25611 pretreated with 700 μM of DX-9065a for 24 h were diluted with GAM broth and were incubated for 24 h, and then the growth was monitored. *P. gingivalis* pretreated with DX-9065a could not grow in GAM broth, but *P. intermedia* pretreated with DX-9065a could (Fig. 2A). We further examined the bactericidal effect of DX-9065a on *P. gingivalis* by measuring RNA contents in the culture. *P. gingivalis* growth was decreased by exposure to DX-9065a within 60 min (Fig. 2B, left). In contrast, RNA content of the supernatants increased in a time-dependent manner (Fig. 2B, right). These results suggest that DX-9065a is bactericidal against *P. gingivalis*.

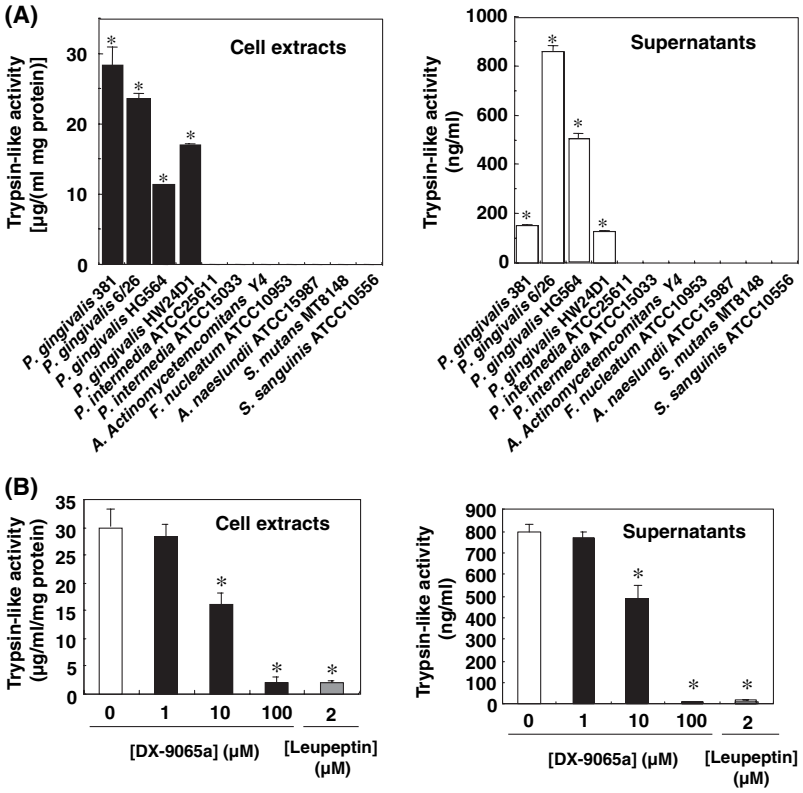

Fig. 3. DX-9065a inhibition of trypsin-like activity in *Porphyromonas gingivalis*. (A) Trypsin-like activity in periodontopathic bacteria cultures. Bacteria were cultured for 24 h, and then the culture supernatants and sediments were harvested. The supernatants and cell extracts were diluted with a buffer containing a protease inhibitor and 100 μM 4-methyl-coumaryl-7-amide (MCA) substrate and then incubated at 37°C for 30 min, and the liberation of MCA was measured by a fluorimeter ($n = 3 \pm$ SD, $^*p < 0.01$ vs. media). (B) DX-9065a inhibits trypsin-like activity in *P. gingivalis* cultures. *P. gingivalis* 6/26 was cultured in Gifu anaerobic medium broth for 24 h, and then the culture supernatants and sediments were harvested. The supernatants and cell extracts were diluted with a buffer containing a protease inhibitor, and DX-9065a or leupeptin was added to the mixture with MCA substrate. The mixture was then incubated at 37°C for 30 min and the liberation of MCA was measured by a fluorimeter ($n = 3 \pm$ SD, $^*p < 0.01$ vs. 0 μM DX-9065a).

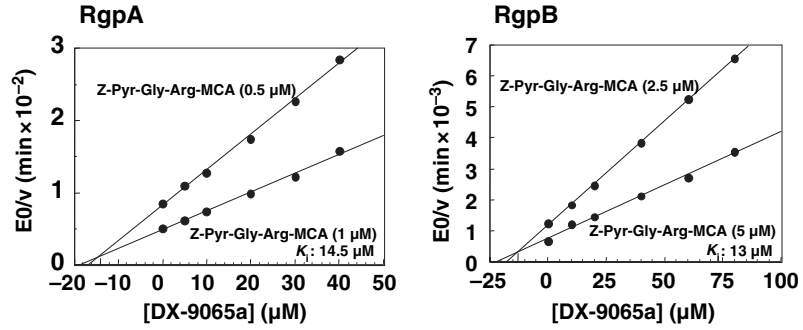

Fig. 4. Inhibition of Rgp amidolytic activity by DX-9065a. DX-9065a at 10, 20, 40, 60 and 80 μM was used for 0.5 nM Rgp. Z-Pyr-Gly-Arg-MCA was used at 0.5 and 1 μM for RgpA and at 2.5 and 5 μM for RgpB, as K_m values of Z-Pyr-Gly-Arg-MCA amidolysis by RgpA or RgpB were 1.7 and 7.5 μM, respectively. K_i values were determined by a Dixon plot. MCA, 4-methyl-coumaryl-7-amide.

DX-9065a blocks trypsin-like activity of *Porphyromonas gingivalis*

We next examined whether the bacteria produced trypsin-like proteinases. Trypsin-like proteinase activity was detected both in cell extracts and culture supernatants of *P. gingivalis* but not of *P. intermedia* ATCC 25611 or *P. intermedia* ATCC 15033 (Fig. 3A). DX-9065a at 100 μM and leupeptin at 2 μM almost completely inhibited the trypsin-like proteinase activities in cell extracts and culture supernatants of *P. gingivalis* in a dose-dependent manner (Fig. 3B).

Inhibition of arginine-specific serine protease amidolytic activity by DX-9065a

To determine whether DX-9065a inhibits the amidolytic activity of purified Rgps, we examined the effect of the amidolytic activity of Rgps on Z-Pyr-Gly-Arg-MCA in the presence of DX-9065a. DX-9065a inhibited the amidolytic activity of Rgps (Fig. 4). However, DX-9065a did not inhibit the amidolytic activity of Kgp, which is a lysine-specific proteinase (not shown). The K_i values of DX-9065a for Z-Pyr-Gly-Arg-MCA amidolysis induced by RgpA and induced by RgpB were 14.5 and 13 μM, respectively (Fig. 4).

Effect of DX-9065a on trypsin-like activity in gingival crevicular fluids

To determine whether DX-9065a is also effective for 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 (113.5 ± 57.2 ng/site vs. 8.9 ± 10.1 ng/site) (Fig. 5A). Furthermore, 10 and 100 μM DX-9065a inhibited the activity in gingival crevicular fluid samples from diseased sites ($n = 6$, % inhibition: 65.2 ± 12.7 and 92.0 ± 7.1, respectively) (Fig. 5B). These results suggest that DX-9065a inhibits activities of Rgps derived from *P. gingivalis* in periodontal pockets.

Discussion

The major finding of this study is that factor Xa inhibitor DX-9065a inhibits the growth of *P. gingivalis* selectively through inhibiting activity of arginine-specific trypsin-like proteinases (Rgps) produced by that microbe. Furthermore, DX-9065a inhibits the trypsin-like activity in human gingival crevicular fluids, which is mostly derived from *P. gingivalis* Rgps. Our findings suggest that DX-9065a may be useful as a new drug for treatment of adult periodontitis.

We have shown that DX-9065a, a potent and selective FXa inhibitor with inhibitory effects on various serine proteases at high concentrations, inhibits the growth of *P. gingivalis* in a biphasic manner. The first phase may be mainly due to inhibition of Rgp activity because K_i values of DX-9065a against RgpA and B were 14.5 μM and 13.0 μM, respectively. However, the reason for the inhibition of the second phase is not yet clear.

Rgps is crucial for *P. gingivalis* growth, as the bacterium is asaccharolytic and is totally dependent on amino acids and peptides as a carbon/energy source (2, 3). *P. gingivalis* degrades environmental proteins and uptakes amino acids and peptides as a carbon/energy source using 'trypsin-like' proteases, such as Rgps. The substrate specificity of Rgp seems to be very similar to that of FXa. Rgp and FXa cleave in common the arginine bond in the peptides, suggesting that these enzymes recognize the amidino group of arginine in the proteins. DX-9065a has two amidino groups in the molecular and competitive inhibitor against RgpA and RgpB but not in that against Kgp. These findings indicate that DX-9065a may bind in the vicinity of the substrate binding site on the molecular of RgpA and RgpB. As shown in Fig. 1(A), DX-9065a inhibits proteolytic activity of Rgp, and *P. gingivalis* could therefore not grow in GAM broth. However, *P. intermedia* growth was also inhibited by DX-9065a, although this bacterium does not produce Rgps. These results imply that an inhibitory mechanism of the compound besides inhibition of Rgps exists. *P. intermedia* produces various serine proteases, such as elastolytic serine protease (10), immunoglobulin-degrading proteases (11), and hemolysin (12). DX-9065a inhibits various

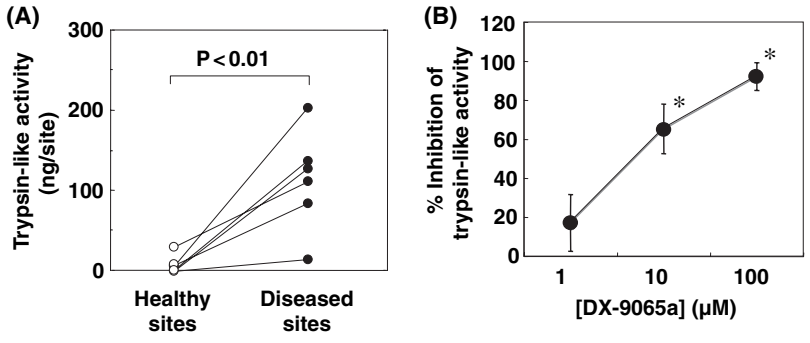

Fig. 5. Effect of DX-9065a on trypsin-like activity in gingival crevicular fluids. (A) Trypsin-like activity in gingival crevicular fluids. Gingival crevicular fluids were collected from one diseased site (●) and one normal site (○) in six patients with adult periodontitis, and the level of trypsin-like activity in each sample was measured by MCA (4-methyl-coumaryl-7-amide) liberation from a substrate as described in the text. (B) Inhibitory effect of DX-9065a on trypsin-like activity in gingival crevicular fluids. Gingival crevicular fluids were collected from eight diseased sites in six patients with adult periodontitis. Various concentrations (1–100 μM) of DX-9065a were mixed with each sample, and then MCA liberation from the substrate was measured as described in the text. The degree of inhibition was expressed as percentage inhibition of trypsin-like activity ($n = 6$ ± SD, $^*p < 0.01$ vs. 0 μM DX-9065a).

serine proteases at high concentrations (more than 100 μM) (7). Therefore, DX-9065a may inhibit serine proteases associated with *P. intermedia* growth. Additional studies are required to reach a final conclusion on this issue.

Various antimicrobial drugs such as tetracycline and penicillin exist and are used for treatment of periodontal diseases (13, 14). However it has been reported that antibiotic resistance in the subgingival flora to these drugs has increased (15). These recent problems related to antibiotic resistance demand further alternatives and effective antimicrobial therapies. It has also been reported that *P. gingivalis* is an important pathogen of refractory periodontitis (16, 17). Furthermore, DX-9065a has no toxic effects on mammalian cells and thrombosis at low concentrations but is effective for *P. gingivalis*; less than 1 mM DX-9065a was not toxic to human gingival fibroblasts (LD_{50} is 12.4 m μM (data not shown) and intravenous administration of 1 m μM DX-9065a did not affect bleeding time in rats (18). Therefore, DX-9065a may be useful as a new drug for treatment of periodontitis, which is strongly associated with *P. gingivalis* infection.

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