Suppression of Pathogenicity of *Porphyromonas gingivalis* by Newly Developed Gingipain Inhibitors

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ABSTRACT

Arg-gingipain (Rgp) and Lys-gingipain (Kgp) are cysteine proteinases produced by *Porphyromonas gingivalis*, a major etiological bacterium of periodontal diseases. Here we show a series of small peptide analogs able to inhibit either Rgp or Kgp, which are synthesized on the basis of the cleavage site specificity of human salivary histatins by each enzyme. Among this series of compounds, carbobenzoxy-Lys-Arg-CO-Lys-N-$(CH_3)_2$ (KYT-1) and carbobenzoxy-Glu(NHN(CH_3)Ph)-Lys-CO-NHCH$_2$Ph (KYT-36) were found to be the most potent inhibitors of Rgp and Kgp, respectively, with K_i values of 10^{-11} to 10^{-10} M order. Both inhibitors exhibited slight or no inhibition on mammalian proteinases such as trypsin and cathepsins B, L, and H. All of the virulence induced by the culture supernatant of *P. gingivalis* tested, including the degradation of various host proteins such as human type I collagen, immunoglobulins, fi-

bronectin, and fibrinogen, disruption of the bactericidal activity of polymorphonuclear leukocytes, and enhancement of the vascular permeability, were strongly inhibited by a combined action of both inhibitors. The functions essential for the bacterium to grow and survive in the periodontal pocket, such as coaggregation and acquisition of amino acids, were also strongly inhibited by the combined action of both inhibitors. The disruption of the adhesion and viability of human fibroblasts and hemagglutination by the organism were strongly suppressed by a single use of KYT-1. These results thus indicate that the newly developed KYT-1 and KYT-36 both should provide a broader application in studies of this important class of enzymes and facilitate the development of new approaches to periodontal diseases.

Periodontal disease is a common inflammatory oral disease characterized by acute progressive lesions of periodontal tissues, excessive leukocyte infiltration, and occurrence of a characteristic microflora. Recent epidemiological studies have demonstrated a strong association between periodontal disease and serious systemic diseases such as atherosclerosis and coronary heart diseases (DeStefano et al., 1993; Beck et al., 1999), diabetes (Teng et al., 2002), pneumonia (Scannapieco, 1999), multiple sclerosis (Shapira et al., 2002), and preterm birth and low birth weight (Romero et al., 2002; Jeffcoat et al., 2003). A marked increase in the prevalence of periodontal disease over the past three decades has thus

prompted efforts to characterize its pathogenesis and to develop new approaches to the therapy.

Porphyromonas gingivalis, a Gram-negative, black-pigmented, asaccharolytic, and anaerobic bacterium, has been strongly implicated in the etiology of some types of periodontitis including chronic adult periodontitis (Slots et al., 1986; Holt et al., 1988). This bacterium produces a novel class of cysteine proteinases referred to as gingipains in both cell-associated and secretory forms. Gingipains consist of arginine-specific cysteine proteinases (Arg-gingipain, Rgp) and lysine-specific cysteine proteinase (Lys-gingipain, Kgp) (Potempa et al., 1995; Kadowaki et al., 2000). We have shown previously with various *P. gingivalis* mutants deficient in Rgp- and/or Kgp-encoding genes that both enzymes play critical roles in most of the virulence of the bacterium (Nakayama et al., 1995; Oka-

ABBREVIATIONS: Rgp, Arg-gingipain; Kgp, Lys-gingipain; KYT-1, carbobenzoxy-Lys-Arg-CO-Lys-N(CH_3)$_2$; KYT-36, carbobenzoxy-Glu(NHN(CH_3)Ph)-Lys-CO-NHCH$_2$Ph; KYT-2, carbobenzoxy-Lys-Arg(NO_2)-CO-Lys-N(CH_3)$_2$; KYT-8, carbobenzoxy-Lys-Arg-H; KYT-22, carbobenzoxy-Glu-Lys-CO-Asn-N(CH_3)$_2$; KYT-26, carbobenzoxy-Glu-Lys-CO-NHCH$_2$Ph; KYT-30, carbobenzoxy-Gln-Lys-CO-NHCH$_2$Ph; BHI, brain heart infusion; BSA, bovine serum albumin; CL, chemiluminescence; MCA, 4-methyl-7-coumaryl-amide; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; α-KG, α-ketoglutarate; DMEM, Dulbecco's modified Eagle's medium; A71561, 1-(3-phenylpropionyl)piperidine-3(R,S)-carboxylic acid-[4-amino-1(S)-(benzothiazole-2-carbonyl)butyl]amide.

moto et al., 1998; Shi et al., 1999; Baba et al., 2001, 2002). These include the destruction of periodontal tissues, the disruption of host defense mechanisms, and the loss of the adhesion activity and viability of human fibroblasts and endothelial cells. Moreover, these enzymes are shown to be essential for the bacterium to grow and survive in the periodontal pocket: they play a critical role independently or cooperatively in the processing of various cell-surface and secretory proteins of *P. gingivalis* (Nakayama et al., 1996; Kadowaki et al., 1998), hemagglutination, coaggregation, hemoglobin binding, and acquisition of heme and amino acids by the bacterium (Nakayama et al., 1998; Okamoto et al., 1998). Both enzymes also seem to contribute to the host defense evasion of *P. gingivalis* because endogenous protease inhibitors, such as serpins, cystatins, and tissue inhibitors of metalloproteinases, have little or no effect on their proteolytic activities (Kadowaki et al., 1994; Abe et al., 1998). These findings thus indicate that potent inhibitors of gingipains should be useful tools both to assess the contribution of their proteolytic activities to the virulence of the bacterium and to facilitate the development of new therapeutic approaches to periodontal diseases.

In this study, we designed and synthesized a series of peptide analogs able to inhibit either Rgp or Kgp on the basis of the cleavage site specificity of histatins by each enzyme. Histatins are a family of histidine-rich polypeptides secreted by human and subhuman primate salivary glands and believed to be important components of the nonimmune defense system in oral cavity (Oppenheim et al., 1988). They have a unique homologous structure containing seven histidine residues and exhibit antimicrobial and antifungal activities (Mackay et al., 1984; Xu et al., 1991). Among this series of compounds, we found that KYT-1 and KYT-36 had the most potent and selective inhibitory activities of Rgp and Kgp, respectively. We also demonstrate that these inhibitors are useful in assessing to what extent the proteolytic activities of Rgp and Kgp contribute to biological activities of *P. gingivalis*.

Materials and Methods

Bacterial Strains and Culture Conditions. *P. gingivalis* ATCC33277 and *Actinomyces viscosus* NY-1 were grown in broth-enriched brain heart infusion (BHI) (37 g/l) (Difco, Detroit, MI) supplemented with yeast extract (5 g/l), hemin (5 mg/l), vitamin K_1 (1 mg/l), and cysteine (1 g/l) under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2). As a defined minimal medium to monitor the growth of *P. gingivalis*, we used α-ketoglutarate/bovine serum albumin (α-KG/ BSA) medium (Milner et al., 1996).

Human Fibroblast Culture. Human gingival fibroblast cell line, Gin-1, was obtained from Dainippon Pharmaceutical Co. (Tokyo, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nisssui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 0.1% $NaHCO_3$, 10% fetal bovine serum (Filtron, Melbourne, Australia), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in humidified 10% CO_2 at 37°C.

Preparations of the Culture Supernatant of *P. gingivalis* and Gingipains. Overnight culture of *P. gingivalis* ATCC33277 in enriched BHI medium was harvested by centrifugation at 10,000*g* for 20 min at 4°C. Ammonium sulfate was added to the supernatant to give 75% saturation. The precipitated proteins were collected by centrifugation at 10,000*g* for 20 min at 4°C and suspended in 10 mM sodium phosphate buffer, pH 7.0. After dialysis against the same

buffer, the insoluble materials were removed by centrifugation at 27,000*g* for 30 min. The resulting supernatant was used as the bacterial culture supernatant. Rgp (Kadowaki et al., 1994) and Kgp (Abe et al., 1998) were purified from the culture supernatants of *P. gingivalis* ATCC33277 and KDP112, respectively, as described previously. Cathepsins B (Towatari et al., 1976), L (Bando et al., 1986), and H (Towatari et al., 1978) each were purified according to the methods described previously.

Enzyme and Inhibition Assays. Proteolytic activities of Rgp and Kgp were determined with the synthetic substrates carbobenzo-xy-Phe-Arg-4-methyl-7-coumaryl-amide (MCA) and carbobenzoxy-His-Glu-Lys-MCA, respectively (both from Peptide Institute, Inc., Osaka, Japan) (Kadowaki et al., 1994; Abe et al., 1998). In brief, appropriate amounts of purified Rgp or Kgp, as well as the bacterial culture supernatant, were added to the reaction mixture (1 ml) containing 5 mM cysteine, 20 mM sodium phosphate buffer, pH 7.5, and 10 μM each fluorogenic substrate. After 10-min incubation at 40°C, the reaction was terminated by adding 100 mM sodium acetate buffer, pH 5.0, containing 10 mM iodoacetic acid (1 ml). The released 7-amino-4-methylcoumarine was measured at 460 nm (excitation at 380 nm) by fluorescence spectrophotometer Hitachi F-3010 (Hitachi Software Engineering Yokohama, Japan). Cathepsins B and L were assayed with carbobenzoxy-Phe-Arg-MCA and cathepsin H with Arg-MCA as described previously (Barrett and Kirschke, 1981). In brief, the reaction mixtures (1 ml) containing 10 mM dithiothreitol, 1 mM EDTA, and 20 mM sodium phosphate buffer, pH 6.0, were incubated at 40°C for 10 min with 10 μM of each fluorogenic substrate. Other details were the same as described for gingipains. For the inhibition assay, the enzymes were preincubated with various concentrations of each inhibitor at 37°C for 5 min before the addition of substrates. All inhibitors were dissolved in dimethyl sulfoxide and used as a final concentration of 0.1%.

Determination of K_i Values. K_i values were determined with appropriate concentrations of substrates and inhibitors. The reaction mixture was incubated and continuously monitored at 40°C for 10 min in the presence or absence of various inhibitors by fluorescence spectrophotometer (Hitachi F-2000). The results were fit to the linearization methods of Lineveawer-Burk, Hanes-Woolf, and Eadie-Hofstee, as determined by computer analyses using Enzyme Kinetics!Pro (ChemSW, Inc., Fairfield, CA). The type of inhibition was identified by statistical comparison of residual sum of squares and Akaike's information criterion. K_i values were estimated according to the inhibition model that gave the lowest Akaike's information criterion value or sum of squares.

Degradation of Histatins by Rgp and Kgp. Human histatins 1, 3, and 5 were kind gifts from Dr. Sugiyama in Okayama University School of Dentistry (Okayama City, Japan). Each histatin (100 μg) was incubated with purified Rgp (0.01–10 μg) or the mixture of purified Rgp and Kgp (0.1 μg each) in 20 mM sodium phosphate buffer containing 5 mM cysteine, pH 7.5, at 37°C for 10 min. The reaction products were separated by a reversed-phase high-performance liquid chromatography on a 15C18-AM column (Nacalai Tesque, Kyoto, Japan) followed by NH_2-terminal amino acid sequence analysis by use of an automatic protein sequencer (model 476A; Applied Biosystems, Foster City, CA).

Degradation of Host-Derived Proteins by the Bacterial Culture Supernatant. Human fibronectin, fibrinogen, α2-macroglobulin, and γ-globulin (10 μg of protein each) were incubated with *P. gingivalis* culture supernatant (0.6 μg of protein) in 20 mM sodium phosphate buffer containing 1 mM dithiothreitol, pH 7.5, at 37°C for 1 h in the presence or absence of KYT-1 and/or KYT-36. Likewise, human acid-soluble type I collagen (10 μg; Seikagaku Kogyo Co., Tokyo, Japan) was incubated with the bacterial culture supernatant (0.6 μg of protein) at 25°C, pH 7.5, for 1 h in the same buffer. All of the reactions were terminated by adding an inhibitor cocktail containing leupeptin, tosyl-L-phenylalane chloromethyl ketone, and tosyl-L-lysine chloromethyl ketone (0.1 mM each). The samples were then applied to SDS-polyacrylamide gel electrophore-

sis in a 5 to 15% polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue R-250.

Luminol-Dependent Chemiluminescence Response. Chemiluminescence (CL) response of PMNs was measured according to the method described previously (Kadowaki et al., 1994). Sterilized oyster glycogen (Sigma-Aldrich, St. Louis, MO) in saline (0.2%) was intraperitoneally injected into guinea pigs. After 14 h, the peritoneal PMNs were collected, washed, and suspended in Hanks' balanced salt solution (1×10^7 cells/ml, more than 90% = PMNs) and then preincubated with the *P. gingivalis* culture supernatant at 37°C for 20 min. The cells were subsequently washed twice with phosphate-buffered saline (PBS) and resuspended in the same buffer at the concentration of 2×10^7 cells/ml. Zymosan A (Sigma-Aldrich) suspended in PBS (20 mg/ml) was boiled for 5 min, washed, and opsonized with guinea pig serum at 37°C for 30 min. The reaction mixtures consisting of 0.1 ml of luminol solution (0.2 mM), 0.1 ml of PMN suspension (2×10^7 cells/ml), and 0.1 ml of zymosan A (20 mg/ml) in 96-well plates were maintained in an automatic luminescence analyzer, MicroLumat Plus (Berthold Technologies, Bad Wildbad, Germany). The intensity of luminescence was automatically recorded for 30 min, and the maximal values were compared.

Vascular Permeability Enhancement Reaction. *P. gingivalis* culture supernatant (15 μg of protein) preincubated with or without inhibitors was intradermally injected into guinea pig back skin. After 30 min, Evans blue (5% in PBS) was injected intravenously. Then, at 20 min, the extravasated dye was quantified by a densitometric analyzer of Science Lab 99 Image Gauge (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Hemagglutination Assay. The 2-day culture of *P. gingivalis* was centrifuged, washed, and resuspended in PBS at an optical density of 0.4 at 540 nm. The bacterial suspensions were preincubated at 37°C for 10 min with or without proteinase inhibitors. Then the erythrocyte suspension (2.5% in PBS) were added and incubated in a round-bottomed microtiter plate at room temperature for 3 h.

Coaggregation Assays. The 2-day culture of the bacterial cells was harvested, washed twice with PBS, and resuspended in a coaggregation buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM CaCl$_2$, 0.1 mM MgCl$_2$, 0.02% NaN$_3$, and 0.15 M NaCl). The suspension was adjusted to a final optical density of 1.0 at 550 nm. *P. gingivalis* suspension (0.35 ml) was preincubated at 37°C for 30 min with or without various proteinase inhibitors and then mixed with an equal volume of *A. viscosus* suspension and incubated at 37°C for 1 h. Coaggregation between the two bacteria was monitored by decrease in an optical density at 550 nm (Cisar et al., 1979).

Cell Adhesion Assay. Human gingival fibroblast Gin-1 cells were plated on eight-well chamber slides at a density of 1×10^4 cells/well and preincubated at 37°C for 24 h in DMEM supplemented with 10% fetal bovine serum. The medium was changed to serum-free DMEM containing the culture supernatant of *P. gingivalis* (83 μg of protein/ml) containing 80 nM Rgp and 32 nM Kgp with or without proteinase inhibitors. The reaction mixture was incubated at 37°C for 6 h. The culture medium was removed, and the cells attached to the chamber slides were washed twice with PBS. The cells were counted under a microscope (Baba et al., 2001).

Assessment of Cell Viability. Cell viability was assessed by a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan). Fibroblasts were seeded at a density of 7×10^3 cells/well into 96-well plates (Falcon 3072; BD Biosciences, San Jose, CA) and incubated at 37°C for 6 h with the culture supernatant of *P. gingivalis* with or without proteinase inhibitors. At the end of the incubation, sodium 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium was added to the culture medium. The culture was maintained for an additional 1 h at 37°C in a CO$_2$ incubator. The amount of reduced tetrazolium was measured at 450 nm with a microplate reader (ImmunoMini NJ-2300; Nalge Nunc International, Tokyo, Japan).

Results

Degradation of Histatins by Rgp and Kgp. Salivary histatins are a family of low-molecular-weight histidine-rich polypeptides. Although histatins 1 to 12 have been isolated from human parotid saliva, histatins 1, 3, and 5 comprise 80 to 90% of the total histatins secreted. It has been reported that histatin 5 inhibits the trypsin-like proteinase produced by *P. gingivalis* (Nishikata et al., 1991; Gusman et al., 2001). We thus investigated the interaction of histatins 1, 3, and 5 with Rgp. The three histatins (100 μg) were incubated with different concentrations of Rgp (0.1, 1, and 10 μg), and then the reaction mixtures were applied on a reversed-phase column chromatography. All histatins were efficiently degraded by Rgp in a dose-dependent manner, and the complete degradation was accomplished by 1 μg of Rgp under the conditions used (Fig. 1A). The preferential cleavage sites of these histatins were determined by amino acid sequencing of the degradation products after incubation of histatins with different concentrations of Rgp (0.01, 0.1, and 1.0 μg for each). The sites cleaved by low amounts of the enzymes were defined as the most favored cleavage sites, as indicated by large arrows. Rgp at concentrations of 0.01 and 0.1 μg exclusively cleaved histatins at the Arg^{12}-Lys^{13} bond. Little or no cleavage was observed at the other sites. When a higher amount of Rgp (1.0 μg) was used, the Arg^{22}-Glu^{23}, Arg^6-His^7, and Arg^{11}-Arg^{12} bonds were additionally cleaved. Likewise, low concentrations of Kgp (0.01 and 0.1 μg) cleaved predominantly at the Lys^{17}-His^{18} bond, and 1.0 μg of Kgp additionally cleaved the Lys^5-Arg^6 and Lys^{11}-Arg^{12} bonds. Thus, histatins were efficiently cleaved by Rgp according to the rank order of Arg^{12}-Lys^{13} $\gg$ Arg^{22}-Glu^{23} = Arg^6-His^7 = Arg^{11}-Arg^{12} bonds, whereas those cleaved by Kgp were according to the rank order of Lys^{17}-His^{18} > Lys^5-Arg^6 = Lys^{11}-Arg^{12} bonds (Fig. 1B).

Design of Specific Inhibitors to Rgp and Kgp on the Basis of the Cleavage Site Specificity of Histatins. The findings that the most efficient cleavage of all histatins by Rgp occurs at Arg^{12}-Lys^{13} bond and that the degradation of histatins 3 and 5 by Rgp is more sensitive than that of histatin 1 strongly suggest the importance of the presence of Lys in the P1′ and P2 positions for the efficient cleavage by Rgp. We thus designed and synthesized a series of small peptide analogs containing Arg at the P1 position and Lys at the P1′ and P2 positions as Rgp inhibitors. Among these compounds, KYT-1 exhibited the most potent inhibition to Rgp with an IC$_{50}$ value of 8×10^{-10} M (Fig. 2). KYT-1–derivative compounds KYT-2, KYT-8, and the optical isomer of KYT-1 (KYT-3) inhibited Rgp less than did KYT-1, indicating the importance of Lys in P1′ and P2 positions. On the other hand, the presence of basic amino acids, particularly His, at the P1′ position and Glu in the P2 position seemed important for efficient cleavage of histatins by Kgp. We thus designed and synthesized a peptide analog, carbobenzoxy-Glu-Lys-CO-Lys-N(CH$_3$)$_2$, as the first Kgp inhibitor. Unfortunately, this compound was unstable in water, methanol, and dimethyl sulfoxide. We then synthesized a series of peptides by modifying the amino acid residue in the P2 position of this compound as Kgp inhibitors (Fig. 2). KYT-22 showed a strong inhibitory action on Kgp, with an IC$_{50}$ value of 1×10^{-9} M. KYT-26 and KYT-30 also had the strong inhibitory action on Kgp,

with an IC_{50} value of approximately 4×10^{-10} M, indicating that Lys in the P1′ position and Glu in the P2 position are variable with other amino acid residues. KYT-36, a derivative of KYT-26, exhibited the most potent inhibition to Kgp, with an IC_{50} value of 2×10^{-10} M. We thus conclude that, among this series of compounds, KYT-1 and KYT-36 are the most potent inhibitors for Rgp and Kgp, respectively.

Characterization of KYT-1 and KYT-36. The effects of KYT-1 and KYT-36 on various proteinases were examined. The K_i values for KYT-1 and KYT-36 determined by the Michaelis-Menten equation are shown in Table 1. The K_i value of KYT-1 for Rgp was calculated to be 1.3×10^{-10} M. Inhibition of Kgp, mammalian trypsin, and cathepsins B, L, and H by KYT-1 was approximately 10^4, 10^4, 10^2, 10^2, and 10^6 times less than that of Rgp, respectively, indicating that KYT-1 is a potent and selective inhibitor for Rgp. The K_i value of KYT-36 for Kgp was calculated to be 7.5×10^{-11} M. No appreciable influence on Rgp was detected even when used with 10^{-4} M KYT-36. Inhibition of trypsin and cathepsins B, L, and H by KYT-36 was approximately 10^3, 10^2, 10^3, and 10^6 times less than that of Kgp, respectively, indicating that KYT-36 is a potent and selective inhibitor for Kgp.

Effects of KTY-1 and KYT-36 on Degradation of Human Proteins by *P. gingivalis* Culture Supernatant. Human type I collagen, a major component of periodontal connective tissue, was extensively degraded by *P. gingivalis* culture supernatant (Fig. 3). This degradation was not significantly inhibited by a single use of KYT-1 or KYT-36 at a concentration of 10^{-4} M. However, when the two inhibitors were given together, a complete inhibition of this degradation

was observed. Likewise, degradation of human fibronectin, γ-globulin, α_2-macroglobulin, and fibrinogen by *P. gingivalis* culture supernatant was inhibited strongly by a combination of KYT-1 and KYT-36 but only slightly by a single use of each inhibitor. Efficient degradation of human fibrinogen (Aα, Bβ, and γ subunits) by the bacterial culture supernatant, suggesting its association with the bleeding tendency in periodontal pockets of periodontitis patients, was also completely inhibited by the combined action of KYT-1 and KYT-36. The results thus indicate not only the importance of both Rgp and Kgp in degradation of various host proteins by *P. gingivalis* but also the strong inhibition of their degradation by a combination of KYT-1 and KYT-36.

Effects of KYT-1 and KYT-36 on Disruption of the Bactericidal Activity of PMNs by *P. gingivalis* Culture Supernatant. PMNs are known to play an important role in host defense mechanisms against acute bacterial infections. They show the luminol-dependent CL response by the generation of active oxygen species during the process of phagocytosis (Allen et al., 1972). The extent of CL thus correlates with their bactericidal activity. It has been demonstrated that the CL response is dose-dependently suppressed by the culture supernatant of *P. gingivalis* (Yoneda et al., 1990) and purified Rgp and Kgp (Kadowaki et al., 1994; Abe et al., 1998). The suppression of the CL response by the bacterial culture supernatant was inhibited significantly by either KYT-1 or KYT-36 but strongly by a combination of both inhibitors (Fig. 4A). The inhibition was completed by the combined action of both inhibitors at concentrations of 10^{-6} to 10^{-7} M. These results are consistent with the results obtained with *P. gingivalis* mutants deficient in Rgp and/or
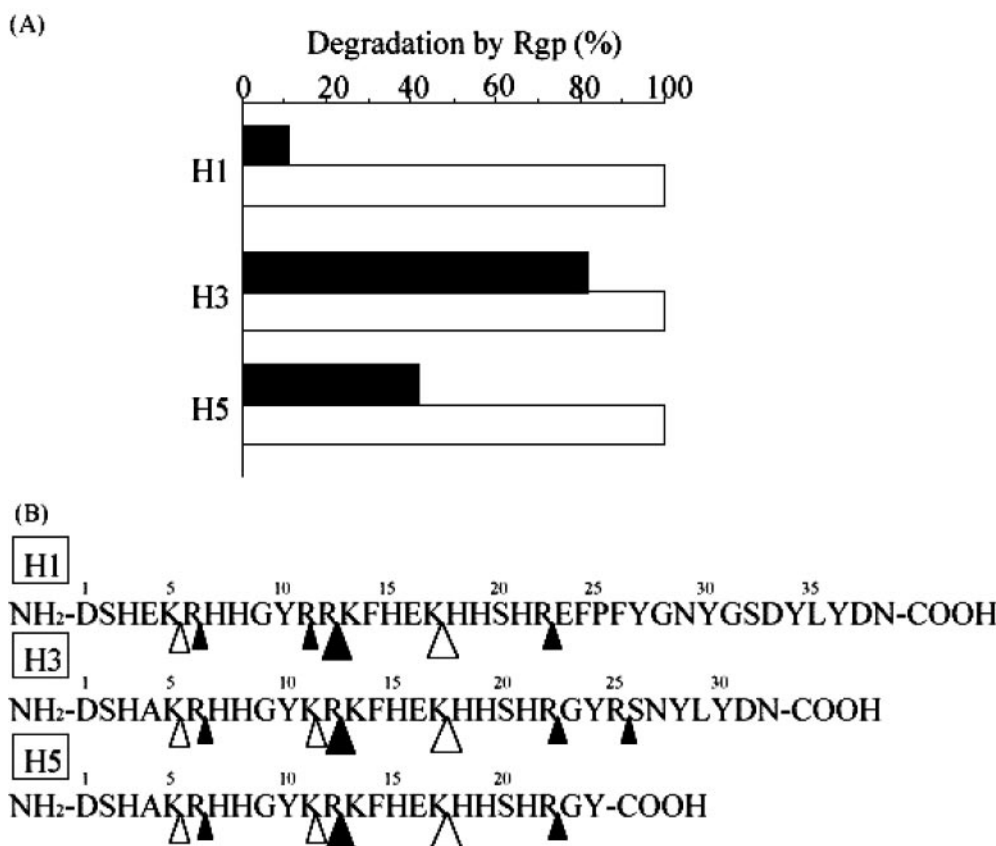

Fig. 1. Degradation of human histatins with Rgp and Kgp. A, degradation of histatins 1, 3, and 5 by Rgp. Each histatin (100 μg) was incubated with 0.1 and 1 μg of Rgp in 20 mM sodium phosphate buffer containing 5 mM cysteine, pH 7.5, at 37°C for 10 min. The reaction products were applied to a reversed-phase column chromatography. The amounts of the intact and degraded histatins were determined by measuring the integrated area of the respective peaks at 280 nm. The values are expressed as percentages of the initial amounts of histatins; ■, 0.1 μg of Rgp; □, 1 μg of Rgp. B, identification of cleavage sites in histatins by Rgp and Kgp. The reaction products of histatins after incubation with either Rgp or Kgp (0.01–1 μg of each) under the same condition as A were applied to a reversed-phase column chromatography followed by analysis of NH_2-terminal amino acid sequence by an automatic protein sequencer. The cleavage sites are indicated as closed (Rgp) or open (Kgp) arrowheads. The size of the arrowhead represents the efficiency of degradation by each enzyme.

Kgp-encoding genes and thus indicate that Rgp and Kgp are responsible for disruption of the bactericidal activity of PMNs by the bacterial culture supernatant.

Effects of KYT-1 and KYT-36 on the Loss of Adhesion and Viability of Human Gingival Fibroblasts by *P. gin-*
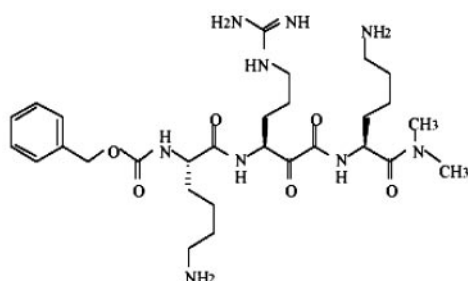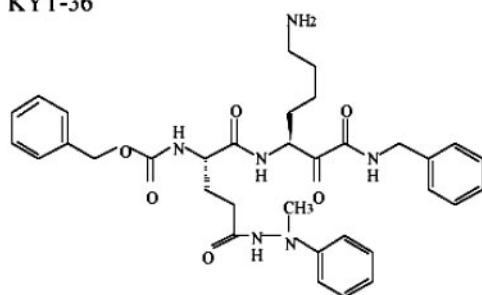

Fig. 2. The structures of KYT-1 and KYT-36.

TABLE 1

K_i values of KYT-1 and KYT-36 toward gingipains and other proteinases

Proteinase	K_i	
	KYT-1	KYT-36
	M	
Rgp	1.3×10^{-10}	$>10^{-4}$
Kgp	2.6×10^{-6}	7.5×10^{-11}
Trypsin	1.2×10^{-6}	1.3×10^{-7}
Cathepsin B	3.4×10^{-8}	3.7×10^{-9}
Cathepsin L	1.2×10^{-8}	7.0×10^{-8}
Cathepsin H	1.5×10^{-4}	1.6×10^{-4}

***givalis* Culture Supernatant.** We reported previously that Rgp was responsible for the loss of adhesion and viability of human gingival fibroblasts (Baba et al., 2001) and umbilical vein endothelial cells (Baba et al., 2002) induced by the culture supernatant of *P. gingivalis*. To assess the ability of KYT-1 and KYT-36 to suppress the disruption of biological activities of fibroblasts by the bacterial supernatant, it was pretreated with these inhibitors and then added to the culture of human gingival fibroblasts. In the absence of these inhibitors, fibroblasts exhibited morphological changes from a spindle shape to a shrunken round shape upon incubation with the bacterial culture supernatant in a time-dependent manner. At 6 h after the incubation, approximately 70% of the cells became detached, and the majority of them (~70%) were induced to cell death (Fig. 4B). However, no significant change in the adhesion activity and the viability of fibroblasts was observed when incubated with the KYT-1–treated bacterial culture supernatant. In contrast, the KYT-36–treated bacterial supernatant showed little or no effect on the loss of the adhesion and viability of fibroblasts. These data are consistent with the results obtained with the *P. gingivalis* mutants deficient in Rgp- and/or Kgp-encoding genes.

In Vivo Effects of KYT-1 and KYT-36 on Vascular Permeability Enhancement by the Culture Supernatant of *P. gingivalis*. It has been demonstrated that Rgp enhances vascular permeability through the activation of prekallikrein and the subsequent bradykinin release (Imamura et al., 1994). We thus determined whether KYT-1 and KYT-36 inhibited the vascular permeability enhancement by the *P. gingivalis* culture supernatant. Intradermal injection of the culture supernatant of *P. gingivalis* into the guinea pig back skin caused significant leakage of Evans blue injected intravenously (Fig. 5). A single administration of KYT-1 or KYT-36 (10^{-6} M each) inhibited the leakage of dye by 40% and 95% of the value obtained with the culture supernatant alone, respectively. The results strongly suggest both the special importance of Kgp in the enhancement of vascular permeability by the bacterium and the actual action of both inhibitors in vivo.

Effects of KYT-1 and KYT-36 on the Bacterial Cell Growth in α-KG/BSA–Defined Medium. The α-KG/BSA–defined medium contains BSA as the sole energy/carbon
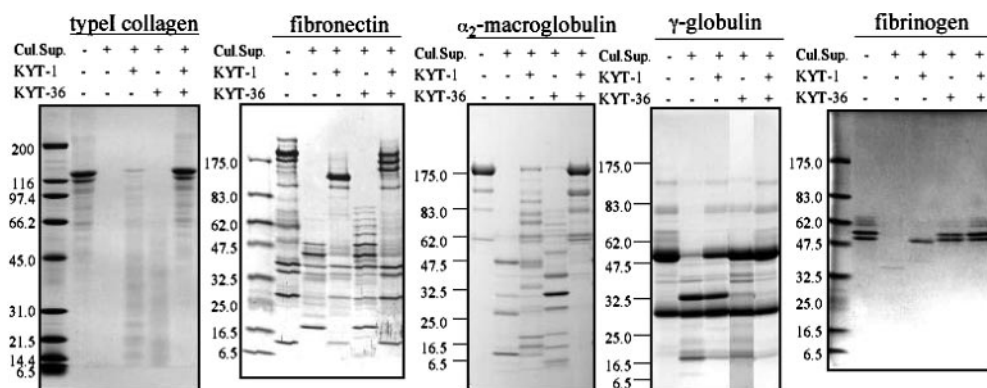

Fig. 3. Effects of KYT-1 and KYT-36 on the degradation of various human proteins by *P. gingivalis* culture supernatant. Human type I collagen, γ-globulin, α2-macroglobulin, fibronectin, and fibrinogen (10 μg of each) were incubated with *P. gingivalis* culture supernatant (0.6 μg of protein) at 37°C (in the case of type I collagen, at 25°C) for 1 h in the absence or presence of 10^{-4} M KYT-1 and/or KYT-36. After incubation, the reaction was terminated by the addition of an inhibitor cocktail containing leupeptin, tosyl-L-phenylalane chloromethyl ketone, and tosyl-L-lysine chloromethyl ketone (final concentration, 0.1 mM each). The samples were then applied to SDS-polyacrylamide gel electrophoresis in 5 to 15% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250.

source and can support the growth of wild-type *P. gingivalis* cells (Milner et al., 1996). We reported previously that the Rgp/Kgp-null (*rgpA rgpB kgp*-deficient) triple mutant of *P. gingivalis* did not grow in this medium (Shi et al., 1999), suggesting that both Rgp and Kgp are essential for the bacterium to acquire peptides and amino acids for their growth through extracellular protein degradation. Thus, we examined the effects of KYT-1 and KYT-36 on the growth of the wild-type *P. gingivalis* in this medium. The growth of *P. gingivalis* was inhibited significantly by KYT-1 and strongly, if not all, by KYT-36 (Fig. 6). This bacterial growth was almost completely inhibited by the combination of KYT-1 and -36. Consistent with the previous observation with the *P. gingivalis* mutants, the present results indicate that the bacterial growth is totally dependent on both Rgp and Kgp and thus is inhibited by a combination of both inhibitors.

Effects of KYT-1 and KYT-36 on Hemagglutination by *P. gingivalis*. Hemagglutination is a distinctive characteristic of *P. gingivalis* that discriminates it from other asacchalolytic black-pigmented anaerobic organisms (Shah and Gharbia, 1989), and it is particularly important for the bacterium to acquire protoheme from hemoglobin for its survival. Previous studies have indicated that the specific genes *rgpA*, *kgp*, and *hagA* of *P. gingivalis* include hemagglutinin domains without proteolytic activities, which are generated by proteolytic processing by Rgp and Kgp (Nakayama et al.,

1996; Kadowaki et al., 1998). This was further substantiated by the experiments showing that Rgp/Kgp-null mutant had no hemagglutinating activity (Shi et al., 1999). To assess the ability and extent of KYT-1 and KYT-36 to inhibit the hemagglutinating activity by *P. gingivalis*, sheep erythrocytes were incubated with the bacterial cells in the presence or absence of these inhibitors. The hemagglutinating activity was strongly inhibited by KYT-1 at 10^{-6} M and completely by its combination with KYT-36 (Fig. 7A). However, little or no effect was observed by a single use of KYT-36. The results indicate that Rgp activity is primarily responsible for hemagglutination by *P. gingivalis*.

Effects of KYT-1 and KYT-36 on the Coaggregation Activity of *P. gingivalis*. *P. gingivalis* is known to coaggregate with other oral microorganisms such as *Actinomyces naeslundii* (Yamaguchi et al., 1998) and *Prevotella intermedia* (Kamaguchi et al., 2001). This coaggregation is important for the bacterium to attach and aggregate in the oral cavity, thereby contributing to the onset and development of periodontal diseases. We thus analyzed the effects of KYT-1 and
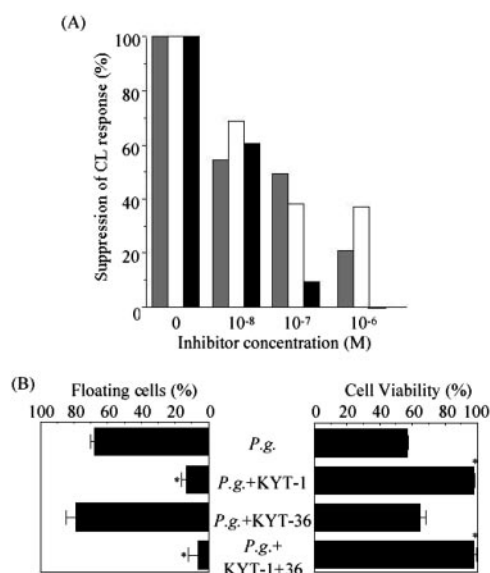

Fig. 4. Effects of KYT-1 and KYT-36 on the disruption of the PMN bactericidal activity (A) and the loss of the adhesion and viability of fibroblasts (B) induced by *P. gingivalis* (*P.g.*) culture supernatant. A, the PMN cell suspension was incubated at 37°C for 20 min with *P. gingivalis* culture supernatant (100 μg of protein) preincubated with or without KYT-1 and/or KYT-36 at 37°C for 5 min. The cells were resuspended in PBS and stimulated with opsonized zymosan A (6.7 mg/ml) in the presence of luminol solution (0.67 mM). The intensity of luminescence was automatically recorded for 30 min. The suppression of CL response (%) = [the peak CL of PMNs incubated with PBS − the peak CL of PMNs incubated with *P. gingivalis* culture supernatant with inhibitor(s)] × 100/[the peak CL of PMNs incubated with PBS − the peak CL of PMNs incubated with *P. gingivalis* culture supernatant without inhibitor(s)]. ▨, KYT-1; □, KYT-36; ■, the combination of KYT-1 and KYT-36. B, human gingival fibroblasts were incubated with the culture supernatant of *P. gingivalis* at a final protein concentration of 83 μg/ml (containing 80 nM Rgp and 32 nM Kgp) with or without KYT-1 and/or KYT-36 at 37°C for 6 h. Then the adhesion and viability were measured as described under *Materials and Methods*.
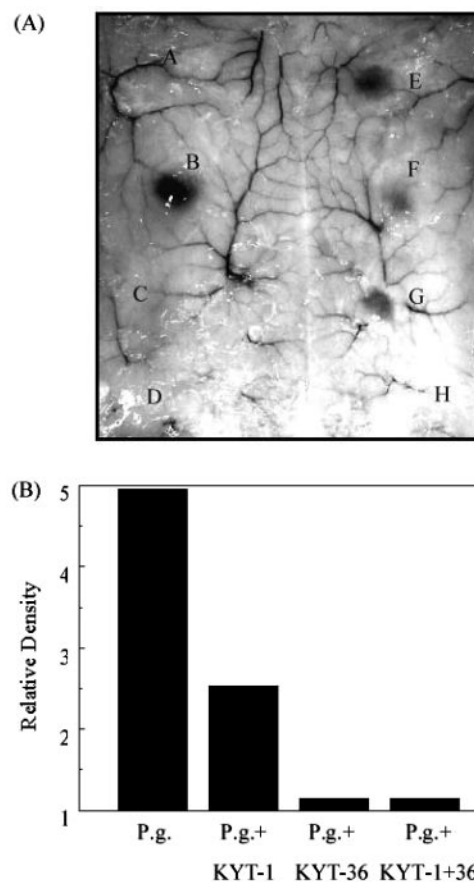

Fig. 5. Effects of KYT-1 and KYT-36 on the vascular permeability enhancement by *P. gingivalis* (P.g.) culture supernatant. *P. gingivalis* culture supernatant (15 μg of protein) was preincubated with or without KYT-1 and/or KYT-36 (10^{-6} M each) and then intradermally injected into the guinea pig back skin. Evans blue dye (5% in PBS) was injected intravenously 30 min after this intradermal injection. After 20-min incubation, the extravasated dye was compared (A). After the densitometric quantification, the data were expressed as the relative ratio of the density to that with PBS (B). A, PBS only; B, *P. gingivalis* culture supernatant; C, KYT-1 alone; D, KYT-36 alone; E, *P. gingivalis* culture supernatant with KYT-1; F, *P. gingivalis* culture supernatant with KYT-36; G, *P. gingivalis* culture supernatant with both KYT-1 and KYT-36; H, KYT-1 and KYT-36.

KYT-36 on coaggregation between *P. gingivalis* and *A. viscosus*. After preincubation at 37°C for 30 min with or without these inhibitors, *P. gingivalis* was mixed with *A. viscosus* at
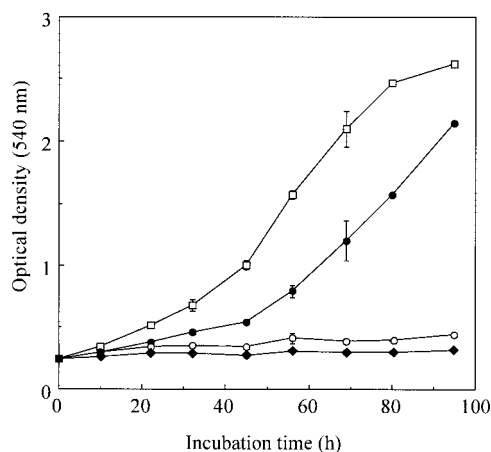

Fig. 6. Effects of KYT-1 and KYT-36 on the growth of *P. gingivalis*. An overnight culture of *P. gingivalis* in enriched BHI broth was diluted 10-fold with α-ketoglutarate/BSA medium supplemented with or without KYT-1 and/or KYT-36 (10^{-4} M each) and incubated anaerobically at 37°C. Growth was monitored by measuring the optical density at 540 nm. □, no inhibitors; ●, KYT-1; ○, KYT-36; ◆, the combination of KYT-1 and KYT-36
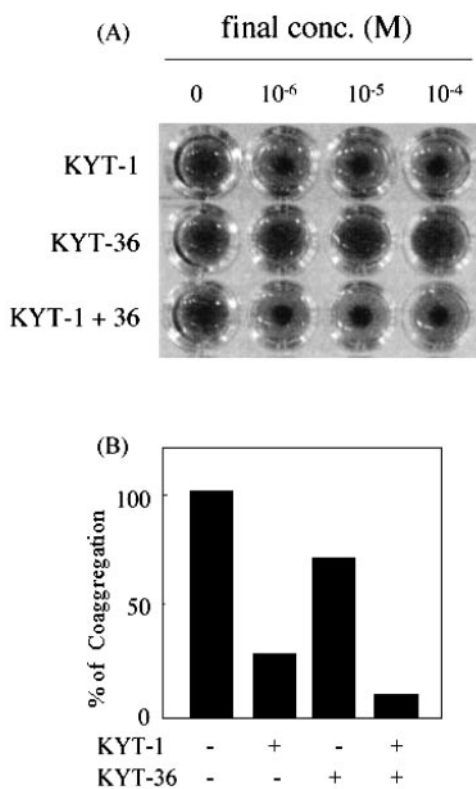

Fig. 7. Effects of KYT-1 and KYT-36 on the hemagglutinating (A) and coaggregating (B) activities of *P. gingivalis*. A, *P. gingivalis* suspension in PBS was preincubated with or without KYT-1 and/or KYT-36 at 37°C for 10 min at the indicated concentrations. Then equal volumes of erythrocyte suspension (2.5% in PBS) were added and incubated in a round-bottomed microtiter plate at room temperature for 3 h. B, *P. gingivalis* suspension was preincubated with or without KYT-1 and/or KYT-36 (10^{-4} M each) at 37°C for 30 min, mixed with equal volume of *A. viscosus*, and incubated for 1 h at room temperature. Coaggregation was monitored by measuring optical density at 550 nm, and represented as the percentage of coaggregation without inhibitors.

approximately equal concentrations, and the extent of coaggregation between the two species was monitored by optical density at 550 nm. Coaggregation induced upon incubation of the two bacterial species for 1 h at room temperature was inhibited significantly by a single use of KYT-1 (>70%) and KYT-36 (>25%) and strongly by the combined action of both inhibitors (>90%) (Fig. 7B). These results indicate that both inhibitors are useful for prevention of the coaggregation of *P. gingivalis* with *A. viscosus*.

Discussion

Previous reports from our and other laboratories have suggested that Rgp and Kgp are involved in a number of intrinsic and extrinsic functions that are associated with the virulence and survival of *P. gingivalis*. These include the bacterial adhesion to host tissue components, processing of bacterial cell-surface and secretory proteins, colonization, hemagglutination, acquisition of heme and amino acids, and destruction of host tissues and cells by the organism. Thus, potent inhibitors for both enzymes would be effective against *P. gingivalis* infection, and both should provide a broader application in studies of this important class of enzymes and facilitate the new treatments of periodontal diseases. Several inhibitors are known to inhibit gingipains. The (acyloxy)methane inhibitor carbobenzoxy-Phe-Lys-CH$_2$OCO-2,4,6-Me$_3$-Ph and the peptidyl chloromethanes D-Phe-Pro-Arg-CH$_2$Cl and D-Phe-Phe-Arg-CH$_2$Cl were reported as specific and rapid inhibitors of Kgp and Rgp, respectively, which could be used for the titration of active sites in the respective enzymes (Potempa et al., 1997). A71561 is also demonstrated to be a reversible inhibitor of Kgp, which blocks the growth, pigmentation, and hemolytic activity of *P. gingivalis* at 0.1 mM (Curtis et al., 2002). The efficacy of these inhibitors in vivo, however, remains to be established. In this study, we designed and synthesized a series of small peptide analogs able to inhibit either Rgp or Kgp, which contain the specific cleavage positions of histatins.

In this study, we provide the first evidence that the reduced proteolytic activities of Rgp and Kgp purified from the culture supernatant of *P. gingivalis* by histatins 1, 3, and 5 are mediated by efficient cleavage of these polypeptides by these enzymes. We demonstrated that Rgp primarily cleaved the Arg^{12}-Lys^{13} bond in histatins, with a minor cleavage at Arg^{22}-Glu^{23}, Arg^6-His^7, and Arg^{11}-Arg^{12} bonds, whereas Kgp efficiently cleaved the Lys^{17}-His^{18} bond in histatins, with a minor cleavage of the Lys^5-Arg^6 and Lys^{11}-Arg^{12} bonds. Biochemical analysis of a series of peptide analogs synthesized revealed that the Lys-Arg-Lys and the Glu-Lys sequences were essential for potent and selective inhibition of Rgp and Kgp, respectively. Ultimately, we designed and synthesized KYT-1 and KYT-36 as the most potent inhibitors specific for Rgp and Kgp, respectively.

Treatment of the culture supernatant of *P. gingivalis* with both KYT-1 and KYT-36 with concentrations of 10^{-6} to 10^{-4} M resulted in strong inhibition of the degradation of various host proteins and the disruption of the bactericidal activity of PMNs. The loss of adhesion and cell viability of fibroblasts by the culture supernatant of *P. gingivalis* was completely inhibited by KYT-1 alone, whereas the enhancement of vascular permeability was inhibited strongly by KYT-36 and significantly by KYT-1. The inhibition of the vascular permeability enhancement by these inhibitors indicates their

efficacy against the bacterial virulence in vivo. In addition, we did not find any toxicity of these inhibitors in the in vivo or in vitro cellular system at the doses tested (i.e., up to 10^{-5} M in vivo and 10^{-4} M in vitro). Furthermore, physiological functions of *P. gingivalis* that are associated with its growth and survival in the periodontal pocket, including coaggregation, hemagglutination, and acquisition of heme and amino acids, were strongly blocked by the combined action of both inhibitors. It is thus concluded that KYT-1 and KYT-36 are useful for the inhibition of a wide range of virulence of *P. gingivalis*.

In summary, the present study is the first example in which potent inhibitors specific for Rgp and Kgp suppress the intrinsic and extrinsic functions associated with the virulence of *P. gingivalis* and essential for its survival. Thus, KYT-1 and KYT-36 both should provide insight into the pathogenesis of periodontal diseases and facilitate the development of new therapeutic approaches to periodontal diseases.

Acknowledgments

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