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Arginine-Specific Gingipains from *Porphyromonas gingivalis* Stimulate Production of Hepatocyte Growth Factor (Scatter Factor) through Protease-Activated Receptors in Human Gingival Fibroblasts in Culture

Akiko Uehara,‡ Koji Muramoto,† Takahisa Imamura,‡ Koji Nakayama,§ Jan Potempa,¶¶ James Travis,∥ Shunji Sugawara,* and Haruhiko Takada‡‡

Cysteine proteinases (gingipains) from *Porphyromonas gingivalis* cleave a broad range of in-host proteins and are considered to be key virulence factors in the onset and development of adult periodontitis and host defense evasion. In periodontitis, an inflammatory disease triggered by bacterial infection, the production of hepatocyte growth factor (HGF) is induced not only by various factors derived from the host, such as inflammatory cytokines, but also by bacterial components. In this study we examined the possible enhanced production of HGF produced by human gingival fibroblasts upon stimulation with gingipains. Arginine-specific gingipain (Rgp) caused a marked production of HGF into the supernatant, the induction of HGF expression on the cell surface, and the up-regulation of HGF mRNA expression in a dose-dependent and an enzymatic activity-dependent manner. Because it has been reported that Rgp activated protease-activated receptors (PARs), we examined whether the induction of HGF triggered by Rgps on human gingival fibroblasts occurred through PARs. An RNA interference assay targeted to PAR-1 and PAR-2 mRNA revealed that gingipains-induced secretion of HGF was significantly inhibited by RNA interference targeted PAR-1 and PAR-2. In addition, the Rgp-mediated HGF induction was completely inhibited by RNA interference targeted to PAR-2. These results suggest that Rgps activated human gingival fibroblasts to secrete HGF in the inflamed sites and the mechanism(s) involved may actively participate in both inflammatory and reparative processes in periodontal diseases.

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Human hepatocyte growth factor (HGF) was first purified from the plasma of patients with fulminant hepatic failure as a hepatocyte-specific growth factor (1). Then, the cDNA for human HGF was cloned from a cDNA library of human placenta (2), and the HGF serum levels were measured by ELISA; these are now used in the clinical prognosis of fulminant hepatic failure. HGF is a broad-spectrum, multifunctional cytokine involved in a variety of physiological processes, including tissue development, regeneration, and wound healing (3–5). HGF was found to be the same protein as the scatter factor (SF) (6, 7), which was independently isolated as a secretory product of fibroblasts that dissociates epithelial cells and increases their motility. The Met receptor tyrosine kinase, encoded by the *c-met* proto-oncogene, is the high affinity receptor of HGF/SF, and HGF/SF exhibits various biological activities, such as mitogenic (8, 9), and morphogenic (10, 11), on cells bearing Met, such as epithelial and endothelial cells, and has tumor cytotoxic effects (12). Several cell lines of mesenchymal origin have been shown to produce HGF/SF as a heparin-binding glycoprotein (13). We have previously reported that human gingival fibroblasts also produce HGF, and this production is enhanced by inflammatory cytokines, such as IL-1α, IL-1β, and TNF-α (14). In addition, we also showed that lipoteichoic acid (LTA), which is a widely distributed cell surface amphiphile of Gram-positive bacteria, and IL-1α synergistically stimulated the production of HGF in human gingival fibroblasts in culture (15).

Oral chronic inflammation, i.e., periodontal disease, is one of the major diseases of mankind and is caused by a bacterial infection leading to gingival inflammation, destruction of periodontal tissues, and loss of alveolar bone, culminating in tooth loss (16, 17). *Porphyromonas gingivalis* has been implicated as a principal bacterium not only in adult periodontitis, but also in rapidly progressive periodontitis (16, 17). *P. gingivalis* possesses a number of putative virulence factors, such as LPS, fimbriae, toxic products of metabolism, and proteinases, all of which can enhance this anaerobe to cause the disease either directly or indirectly by the activation of
host cells to release inflammatory mediators (18). It is clear that all the trypsin-like proteinase activities of *P. gingivalis* are due to two types of cysteine proteinases (19). Among them, two kinds of cysteine proteinases specific for Arg-X (50 and 95 kDa) (20) are referred to as arginine-specific gingipain (Rgp) (18), and another type is lysine-specific gingipains (Kgp) (21). The 95-kDa high molecular mass Rgp (HRgpA) differs from the 50-kDa Rgp (RgpB) in that the protein noncovalently complexes with the hemagglutinin/adhesion domain in the same manner as Kgp. It has been shown that gingipains play a critical role in the onset of inflammation through the enhancement of vascular permeability by activation of the kallikrein/kinin pathway (22, 23), dysregulation of plasma clot formation (24–26), activation of complement components (27), and modification of neutrophil function (28). Gingipains are also indispensable for the expression of *P. gingivalis* lipoproteins, an important cell surface component of this bacterium for adhesion and colonization, through normal processing of immature fimbriin (29). Furthermore, we have shown that the gingipains cleave CD14 on human monocytes (30), gingival fibroblasts (31), and ICAM-1 human oral epithelial cells (32), which consequently inhibits CD14-dependent cell activation by LPS and the interaction between epithelial cells and leukocytes, suggesting that *P. gingivalis* could evade immune surveillance triggered by innate immunity. Periodontitis is clinically characterized as inflammation in periodontal connective tissue, in which the dominant cell type is human gingival fibroblasts. Human gingival fibroblasts may actively participate in the inflammatory response by producing various cytokines (33) and chemokines, such as IL-8 (14), which are released from human gingival fibroblasts via CD14 (34).

Protease-activated receptor (PAR) family members are G protein-coupled receptors characterized by a proteolytic cleavage of the N terminus that exposes tethered ligands and autoactivates the receptor function (35–37). There are four members of this family. PAR-1, PAR-3, and PAR-4 are activated mainly by thrombin, and PAR-2 is activated by trypsin and mast cell tryptase as well as coagulation factors VIIa and Xa, but not by thrombin (35–37). Because PARs are expressed in a wide variety of cell types, including neurons, recent studies suggest that PARs play important roles in various pathophysiological processes, including growth, development, inflammation, tissue repair, and pain (35–37). In this context, we demonstrated that neutrophil serine proteinase 3 activates human epithelial cells and human gingival fibroblasts via the PAR-2 pathway (38, 39). Human gingival fibroblasts express PAR-1 and -2, but not PAR-3 and -4 (39). RgpB has been reported to cleave and activate PAR-2 on human neutrophils (40) and to activate PAR-1 and PAR-2, followed by the induction of IL-6 secretion on human oral epithelial cell line KB cells (41), and cause platelet aggregation via PARs (42). More recently, it has been reported that neuropeptide was released from dental pulp cells upon stimulation with RgpB via PAR-2 signaling (43). Taking all the above findings into consideration, we hypothesized that gingipains might activate human gingival fibroblasts to secrete HGF through the PAR pathway.

**Materials and Methods**

**Reagents**

Ph-e-Pro-Arg-chloromethyl ketone (FPR-cmk) was obtained from Bachem Bioscience. Anti-human HGF mAb was purchased from R&D Systems. Anti-human PAR-1 mAb ATAP2 (mouse IgG) raised against aa 42–55 of human PAR-1, anti-human PAR-2 mAb SMA11 (mouse IgG2a) raised against aa 37–50 of human PAR-2, and rabbit anti-human PAR-3 polyclonal Ab raised against aa 1–13 of human PAR-2 were obtained from Santa Cruz Biotechnology. The phospholipase C (PLC) inhibitor, U73122, and its control, U73343, were obtained from Calbiochem-Novabiochem.

**Purification and activation of gingipains**

Two forms of gingipains, 95-kDa HRgpA and 50-kDa RgpB gingipains, were purified from *P. gingivalis* HG66 strain culture supernatant, as described previously (44, 45). The purity of each enzyme was checked using SDS-PAGE. In 10% Tricine gel (Von Jagow) RgpB migrated as a single band with a mobility equivalent to a molecular mass of 48 kDa and homogeneity >95%, as determined using laser densitometry scanning of the gel. HRgpA resolve into four major and one minor band on SDS-PAGE (44). The identity of each protein band was confirmed by N-terminal sequence analysis as being derived from the HRgpA polyprotein. The amount of active enzyme in each purified gingipain was determined by active site titration using FPR-cmk as active site tiritant for Rgps (21). The same inhibitors were used to obtain inactivated gingipains with covalently modified active site cysteine residues. The concentration of fully activated gingipains with cysteine was calculated from the amount of inhibitors needed for complete inactivation of the proteinases. Therefore, the concentration of gingipains indicated in this study was represented as that of active gingipains. To activate gingipains, gingipains were diluted to 10 μM in 0.2 M HEPES, 5 mM CaCl2, and 10 mM cysteine (pH 8), and incubated at 37°C for 10 min. The activated gingipains were then diluted with medium or buffer. To block the enzymic activity of gingipains, activated gingipains were incubated with the specific inhibitor, FPR-cmk, for 10 min at room temperature before use.

**Collection of supernatants of wild-type *P. gingivalis* and rgpB double-mutant *P. gingivalis* strain**

*P. gingivalis* 33277 was anaerobically grown to stationary phase in enriched brain heart infusion broth with menadione and hemin without antibiotics. An Rgp-null mutant strain of *P. gingivalis* KDF133 (rgpB double mutant; rrgB2::erm rrgB2::tetQ) (46) was anaerobically grown to stationary phase in enriched brain heart infusion broth with menadione, hemin, and erythromycin (10 μg/ml). After 4 days of culture, supernatants were dialyzed against PBS and collected by centrifugation at 20,000 × g for 30 min. Three-milliliter portions of supernatants were concentrated to 500 μl by Vivaspin 2 concentrators (Vivaspace) at 6000 × g at 4°C and used as test culture supernatants.

**Cells and cell culture**

Human gingival fibroblasts were prepared from the explants of normal gingival tissues of 6-year-old patients under informed consent given by the parents because of the age of the donors (39). The explants were cut into pieces and cultured in 100-mm diameter tissue culture dishes (Falcon; BD Biosciences) in α-MEM supplemented with 10% heat-inactivated FCS, with a medium change every 3 days for 10–15 days until confluent cell monolayers were formed. To avoid the possibility that trypsinization affects the amounts of PARs and other surface markers, we used Sigma-Aldrich’s CDS. Because CDS contains no protein and allows dislodging of cells without the use of enzymes, cellular proteins are preserved without enzymatic modification or adsorption of foreign proteins. After three to four subcultures with CDS, homogeneous, slim, spindle-shaped cells growing in characteristic swirls were obtained. The cells were used as confluent monolayers at subculture levels 2–7. The experimental procedure was approved by the ethical review board (Tohoku University Graduate School of Dentistry).

**RNA extraction and RT-PCR assay**

Total cellular RNA was obtained using Isogen (Nippon Gene) and was reverse transcribed using random hexamer primers and avian myeloblastosis virus reverse transcriptase XL (Life Sciences), as described previously (39). The primers used for PCR were as follows: HGF: forward, 5’-CGAGGCTCAACGGTGCCAGGAGG-3’; reverse, 5’-GTATGCGAGAAGCTTATCACAAGCTAGG-3’; reverse, 5’-GTGGGCTCCGTCGTGGTGGTGGTGGTCCTAGGAGG-3’; and reverse, 5’-CATGAGGCTCATGAGGCTTGGGACTGATGATG-3’. The primers for HGF and GAPDH were designed to generate fragments of 447 and 983 bp, respectively. PCR was performed for 30 cycles for 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide and photographed under UV light. The bands on the photographs were scanned and then analyzed using an Image Master 1D (Pharmacia Biotech). The results are expressed as the relative mRNA accumulation, corrected with reference to GAPDH mRNA as an internal standard.
Determination of HGF in culture supernatants

Human gingival fibroblasts (2 × 10^4) were seeded into each well of 96-well culture plates in 100 μl of α-MEM supplemented with 10% FCS. After overnight cultivation, the cells were washed, and the medium was changed to α-MEM. Then the cells treated with the given concentrations of gingipains for the indicated times at 7 × 10^5 cells/70 μl in the presence or the absence of the specific inhibitor, FPR-cmk, for 15 min at 37°C. Cultivation was conducted in triplicate, and HGF levels in the culture supernatants were measured using an ELISA kit (Otsuka Pharmaceutical). The concentrations of the cytokines in the supernatants were determined using the Softmax data analysis program (Molecular Devices).

Flow cytometry

Flow cytometric analyses were performed using a FACSCalibur cytomter (BD Biosciences). Human gingival fibroblasts were stimulated with or without Rgps for 24 h at 37°C. After the incubation, cells were collected by nonenzymatic CDS and washed in PBS. Cells were stained with anti-PAR-1 Ab, anti-PAR-2 Ab, or control IgG at 4°C for 30 min, followed by FITC-conjugated goat anti-mouse IgG (BioSource International) at 4°C for an additional 30 min. For PAR-3 staining, cells were incubated with rabbit anti-PAR-3 polyclonal Ab or control IgG for 30 min, followed by FITC-conjugated swine anti-rabbit IgG (DakoCytomation) at 4°C for another 30 min. For HGF staining, intracellular staining was performed. Briefly, the cells were washed with staining buffer, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 15 min at 4°C. Then the cells were incubated with goat anti-human HGF Ab or control IgG for 30 min, followed by FITC-conjugated rabbit anti-goat IgG (DakoCytomation) at 4°C for another 30 min. To calculate the percentage of positive cells, the baseline cursor was set at a channel that yielded <2% of events positive with the isotype Ab control. Fluorescence to the right was counted as specific binding.

Immunostaining

Human gingival fibroblasts were cultured on eight-chamber glass slides (Falcon; BD Biosciences) until confluent, treated with Rgps (200 nM) for 24 h at 37°C in a 5% CO₂ incubator, and washed three times with PBS. After fixation with 4% paraformaldehyde for 15 min at room temperature, the cells were treated with 0.5% Triton X-100 for 15 min and washed with PBS. Cells were then treated with goat anti-human HGF mAb for 3 h at room temperature. Samples were washed and incubated with Alexa Fluor 488 rabbit anti-goat IgG (Molecular Probes). Nuclei were visualized by staining with 4',6'-diamino-2-phenylindole in blue (Molecular Probes). Samples were photographed with an AxioCamMRm digital camera mounted on a Zeiss AxioImager Z1 microscope using the application Zeiss AxioVision 4 software.

Analysis of peptide cleavage

A peptide corresponding to a region spanning the cleavage site of PAR-1, residues 36–48 (K^36ATLPRSFLRNP^48) and PAR-2, residues 30–43 (K^30SSKRLSIGKV^33), respectively, were synthesized by Takara Shuzo. The peptide (200 μM) was incubated with proteases for 30 min at 37°C in 10 mM Tris-HCl (pH 8.0). Each digest was separated by reverse phase HPLC on a TSKgel ODS 120T column (4.6 × 250 mm; Tosoh) using a linear gradient from 0–40% acetonitrile in 0.1% trifluoroacetic acid for 30 min. The amino acid sequences of peptide fragments were analyzed by a gas phase protein sequencer (PPSQ-1; Shimadzu) and a acid for 30 min. The amino acid sequences of peptide fragments were using a linear gradient from 0 – 40% acetonitrile in 0.1% trifluoroacetic (30SSKGRSLIGKVDGT43), respectively, were synthesized by Takara

RNA interference

Transfection for targeting endogenous PAR-1, PAR-2, p65, and lamin A/C was conducted using Lipofectamine 2000 (Invitrogen Life Technologies) and short interfering RNA (siRNA; final concentration, 200 nM), according to the manufacturer’s instructions. The siRNA of PAR-1, PAR-2, and p65 were purchased from Santa Cruz Biotechnology, and the siRNA of lamin A/C was purchased from B-Bridge International, respectively.

NF-κB activity

Activated NF-κB was measured with an NF-κB assay kit specific for the p65 subunit according to the manufacturer’s instructions (Active Motif). Briefly, samples of whole cell extracts (1–10 μg of protein/well) were added to 96-well plates coated with an oligonucleotide containing the NF-κB consensus site (5’-GGGACTTCC-3’) and incubated for 1 h at room temperature with mild agitation. After three washes, NF-κB p65 Ab was added for 1 h without agitation, followed by HRP-conjugated anti-mouse IgG. Colorimetric reactions were developed, stopped, and measured at 450 nm. The specificity of binding was also examined using an oligonucleotide containing a wild-type or mutated NF-κB consensus binding site.

Data analysis

All experiments in this study were performed at least three times to confirm the reproducibility of the results. In most experiments, values are represented as the mean ± SD of triplicate assays. The significance of differences between the two means was evaluated by one-way ANOVA using the Bonferroni or Dunnett method, and values of p < 0.01 were considered significant.

Results

Effects of Rgps on the production and secretion of HGF from human gingival fibroblasts

We first examined the effects of Rgps (HRgpA and RgpB) on the secretion of immunoreactive HGF from human gingival fibroblasts. Incubation of human gingival fibroblasts with 200 nM Rgps resulted in a time-dependent increase in the production of HGF (Fig. 1A). A significant secretion was observed from the 15 min

FIGURE 1. Induction of HGF secretion by Arg-gingipains in human gingival fibroblasts in culture. A, Human gingival fibroblasts were stimulated with 200 nM gingipains (HRgpA and RgpB) for the indicated times in triplicate at 37°C. B and C, Human gingival fibroblasts were stimulated with gingipains at the indicated concentrations for 24 h in triplicate at 37°C. D, Activating solution for gingipains (0.2 M HEPES, 5 mM CaCl₂, and 10 mM cysteine (pH 8.0)) was used as a control. D, Human gingival fibroblasts were cocultured with or without 1, 5, 10, or 20% of test culture supernatants from wild-type P. gingivalis 33277 or Rgp mutant P. gingivalis KDP 133 for 24 h in triplicate at 37°C. HGF levels in the culture supernatants were determined by ELISA and expressed as the mean ± SD. *p < 0.01 vs medium alone. The results are representative of three different experiments.
incubation point, and a marked increase occurred at 24 h. Incubation of the cells in the presence of various concentrations of Rgps for 24 h resulted in a dose-dependent increase in HGF (Fig. 1, B and C). HGF at 200 nM was most effective in stimulating production. RNA was then extracted from gingival fibroblasts stimulated with Rgps, and RT-PCR was performed to define the level of HGF mRNA. HGF mRNA was already weakly expressed in untreated cells, and the expression of HGF mRNA was significantly increased by Rgps (Fig. 2). Up-regulation of HGF mRNA occurred from 6–24 h of culture, and peak induction occurred at 8 h (Fig. 2C). To confirm the induction of HGF expression by Rgps on human gingival fibroblasts, we performed flow cytometry and immunostaining. As shown in Fig. 3, unstimulated cells only slightly expressed intracellular HGF protein, whereas treatment with Rgps induced remarkable expression of HGF protein on human gingival fibroblasts. It must be noted that HGF production is not up-regulated by the solution used for the activation of gingipains.

In addition, the culture supernatants from wild-type P. gingivalis clearly up-regulated HGF production in human gingival fibroblasts in a dose-dependent manner, whereas the culture supernatants from the HRgpA RgpB double-mutant (Rgp-null mutant) strain of P. gingivalis scarcely up-regulated HGF production (Fig. 1D). These results clearly indicated that Rgps, but not other products, are responsible for HGF induction in the cell-free products of P. gingivalis.

**Effect of the Rgp inhibitor on induction of HGF in human gingival fibroblasts stimulated with gingipains**

To confirm that the induction of HGF was due to the enzymatic activity of Rgps, an inhibitor specific for Rgps, as reported by Potempa et al. (21), was used. FPR-cmk, an effective inhibitor of Rgps, efficiently inhibited Rgps-induced HGF secretion (Fig. 4). These results indicated that cell activation caused by Rgps was directly due to the enzymatic activity of gingipains themselves, and not to the enzymatic activity of contaminating components in Rgps.

**FIGURE 2.** Induction of HGF mRNA expression upon stimulation with Arg-gingipains in human gingival fibroblasts. Human gingival fibroblasts were stimulated with 200 nM Arg-gingipains for 8 h (A and B), or for the times indicated (C). Activating solution for gingipains was used as a control. Total RNA was extracted, and the expressions of HGF and GAPDH mRNA were analyzed by RT-PCR. Bands were further quantified using an imaging analyzer (B and C). The results are expressed relative to GAPDH mRNA as an internal standard. Two additional experiments gave results similar to those shown here.

**FIGURE 3.** Induction of HGF on human gingival fibroblasts by Arg-gingipains. Human gingival fibroblasts were incubated with or without 200 nM HRgpA or RgpB for 24 h at 37°C. Activating solution for gingipains was used as a control. The expression of HGF on the cells was analyzed by flow cytometry. For immunostaining, after fixation, cells were treated with anti-HGF mAb, and then visualized with Alexa Fluor 488 (green). Nuclei were visualized by staining with 4′,6′-diamino-2-phenylindole (blue). The results are representative of three different experiments with similar results.

**FIGURE 4.** HGF secretion by Arg-gingipains was inhibited by their specific inhibitor. Gingipains were pretreated with FPR-cmk at the doses indicated for 15 min at 37°C before use. Human gingival fibroblasts were stimulated with or without gingipains for 24 h. Activating solution for gingipains was used as a control. HGF levels in the culture supernatants were determined by ELISA and are expressed as the mean ± SD. *p < 0.01 compared with no inhibitor. The results are representative of three different experiments.
Effects on PAR expression on the cell surface of human gingival fibroblasts by Arg-gingipains

As it has been recently reported that RgpB activated PAR-1 and PAR-2 on human oral epithelial cells (41), we next examined whether HGF induction by Rgps also occurred through PAR family molecules. As mentioned above, human gingival fibroblasts constitutively express PAR-1 and PAR-2, but not PAR-3 and PAR-4, at the mRNA level and as cell surface proteins (39). Treatment with Rgps clearly augmented the expressions of PAR-1 and PAR-2, but not PAR-3, on the cell surfaces of human gingival fibroblasts in culture, determined by flow cytometry (Fig. 5).

Cleavage of human PAR-1 and PAR-2 peptides with exposure of their tethered ligands by Arg-gingipains

The above results, in addition to previous reports (40, 41), led us to examine whether Rgps cleave PAR-1 and/or PAR-2 at a specific site with the exposure of their tethered ligands. To investigate this possibility, a peptide corresponding to the region surrounding the cleavage site of human PAR-1 and PAR-2 was incubated with Rgps, and proteolytic fragments were analyzed. Thrombin and trypsin were used as positive agonists for PAR-1 and PAR-2, respectively. The PAR-1 peptide was rapidly cleaved at the site R41-S35 by 5 nM HRgpA and RgpB or thrombin, but not trypsin (Fig. 6A), and the PAR-2 peptide was also rapidly cleaved at the site R52-S35 by 5 nM HRgpA and RgpB or trypsin, but not thrombin (Fig. 6B). The measured molecular masses of digested peptides (P1, 1613.0; P1-1, 378.9; P1-2, 672.3; P1-3, 636.2; P2, 1404.5; P2-1, 534.2; P2-2, 893.4) were in good agreement with the calculated values (P1, 1613.8; P1-1, 343.3; P1-2, 671.8; P1-3, 634.8; P2, 1404.6; P2-1, 533.6; P2-2, 889.0). These findings definitely demonstrated that Rgps cleaved PAR-1 and PAR-2 with the exposure of their tethered ligands.

Involvement of PAR-1, PAR-2, PLC, and NF-κB in Rgps-induced human gingival fibroblasts

To confirm the involvement of PARs, we used siRNA to block the expression of PARs. Transfection of human gingival fibroblasts with PAR-1- or PAR-2-specific siRNA resulted in the inhibition of PAR-1 or PAR-2 mRNA, but not GAPDH mRNA, from 24 to 72 h of culture (data not shown). In both PAR-1- and PAR-2-siRNA transfected cells, but not in lamin A/C-siRNA-transfected cells, Rgps-induced activation of human gingival fibroblasts was significantly suppressed (Fig. 7). In contrast, HGF productions triggered by PAR-1AP and PAR-2AP were specifically inhibited in PAR-1- and PAR-2-silenced cells, respectively (Fig. 7). In common with many G protein-coupled receptors, the principal mechanism of PAR-mediated activation is through Goq proteins, resulting in the activation of PLC (35–37). To examine whether PLC is also involved in Rgps-induced activation of human gingival fibroblasts, the cells were treated with gingipains in the presence of the PLC inhibitor, U73122, or the control compound, U73343, for 24 h. As shown in Fig. 8A, the inhibition of PLC completely inhibited the production of HGF induced by Rgps.

It has been reported that agonists of PAR-1 and PAR-2 induced the activation of NF-κB (39, 48–50). As shown in Fig. 8B, Rgps clearly activated NF-κB in human gingival fibroblasts. Therefore, we examined the mechanism using siRNA targeting p65, which is a component of the NF-κB pathway. The up-regulation of HGF secretion induced by Rgps was significantly inhibited in p65-silenced, but not lamin A/C-silenced, human gingival fibroblasts (Fig. 8C). These results indicated that HGF secretion induced by Rgps occurred through PLC and NF-κB downstream of PARs signaling.
The regulation of this step is necessary to understand the patho-
step in the HGF-induced signaling pathway, detailed knowledge of
step of generating biologically active HGF is a critical limiting
types of cells, such as endothelial and epithelial cells. Because the
a typical paracrine factor, and Met is also expressed on various
ffects on the dissociation and migration (called scattering) of the
cells bearing Met from their neighbors through its pleiotropic ef-
clearly demonstrated that Rgps (HRgpA and RgpB) cleaved the
arying activity of P. gingivalis is totally attributable to RGPs,
whose the gingipain-null mutant strain were scarcely ac-
this respect (Fig. 1D). The findings indicated that the HGF-
arginase proteins, Arg- gingipains, activated human gingival fibroblasts
to increase the mRNA expression of HGF and provoked a marked
cretion of HGF through PAR-1, PAR-2, and NF-κB. This is the
first report on HGF induction by gingipains. P. gingivalis gingi-
pains have been studied as a possible mechanism for the bioactiv-
ties leading to the virulence factor of this bacterium in relation to
flammation. With respect to gingipain signaling, RgpB has been
found to activate PAR-1, -2, and -4 (40–43). Our present data
clearly demonstrated that Rgps (HRgpA and RgpB) cleaved the
PAR-1-tethered ligand between Arg41 and Ser42 and the PAR-2-
tethered ligand between Arg31 and Ser35 to activate human gingi-
val fibroblasts. Because bacterial components such as gingipains
are inevitably contaminated with other minor bioactive compo-
antly affect experimental results, we tried to exclude the
ossibility that the contaminating components in the Rgp spec-
imens were responsible for cell activation. As a result, cell acti-
vation through PAR-1 and PAR-2 triggered by Rgps was com-
pletely inhibited by treatment with the gingipain-specific inhibitor,
FPR-cmk (Fig. 4). It must be noted that the culture supernatants
from wild-type P. gingivalis up-regulated HGF production,
whereas those from the Rgp-null mutant strain were scarcely ac-
this respect. The gingival tissue is exposed to periodontal bacteria and their
products in culture supernatants are
physiological roles of HGF. In fact, enhanced activation of HGF
has been reported in injured tissues (51).
The gingival tissue is exposed to periodontal bacteria and their
products and, by receiving and transmitting signals, plays an im-
portant role in the overall dialogue that occurs between pathogens
and the host. In periodontitis, an inflammatory disease triggered by
bacterial infection, production of HGF could be induced not only
by various factors derived from the host, such as inflammatory
cytokines, but also by bacterial components. Fimbriae from P. gin-
givalis, which are putative virulence factors that provoke gingivitis
and periodontitis, are inducers of HGF by gingival fibroblasts in
vitro (52). As mentioned above, LTA from Streptococcus sanguis,
a common Gram-positive bacterium in the oral cavity, synergisti-
cally stimulates the production of HGF by gingival fibroblasts with

FIGURE 7. Involvements of PAR-1 and PAR-2 in Arg-gingipains-in-
duced human gingival fibroblasts activation. Human gingival fibroblasts
were transfected with PAR-1-, PAR-2-, and lamin A/C-specific-siRNA for
24 h and incubated for an additional 24 h in the presence or the absence of
gingipains (200 nM) at 37°C. PAR-1AP and PAR-2AP (100 μM) were
used as positive controls. Activating solution for gingipains was used as a
control. The concentrations of HGF in the culture supernatants were
determined by ELISA. *p < 0.01 compared with the respective control. The
results are representative of three different experiments.

Discussion
In the present study we provide evidence that P. gingivalis cysteine
proteinases, Arg- gingipains, activated human gingival fibroblasts
to increase the mRNA expression of HGF and provoked a marked
secretion of HGF through PAR-1, PAR-2, and NF-κB. This is the
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from wild-type P. gingivalis up-regulated HGF production,
whereas those from the Rgp-null mutant strain were scarcely ac-
this respect (Fig. 1D). The findings indicated that the HGF-
inducing activity of P. gingivalis is totally attributable to RGPs,
whereas other P. gingivalis products in culture supernatants are
scarcey active in this respect. HGF-induced signaling could participate in the dissociation
of cells bearing Met from their neighbors through its pleiotropic ef-
fects on the dissociation and migration (called scattering) of the
cells, the production of destructive proteinases, the induction of
antiapoptotic signals that are required to protect the migration cells
from anoikis, cellular proliferation, and angiogenesis (4). HGF is
a typical paracrine factor, and Met is also expressed on various
types of cells, such as endothelial and epithelial cells. Because the
step of generating biologically active HGF is a critical limiting
step in the HGF-induced signaling pathway, detailed knowledge of
the regulation of this step is necessary to understand the patho-

FIGURE 8. Involvements of PLC and NF-κB downstream of PARs in
Arg- gingipain-induced HGF secretion in human gingival fibroblasts. A.
Human gingival fibroblasts were incubated with or without 10 μM U73122
or control U73343 for 30 min. Then the cells were stimulated with gingi-
pains (200 nM) for 24 h. PAR-1AP and PAR-2AP (100 μM) were used as a
positive control. Activating solution for gingipains was used as a control.
Concentrations of HGF in the culture supernatants were determined by
ELISA. B, Human gingival fibroblasts were incubated for 1 h in the pres-
ence or the absence of gingipains (200 nM) at 37°C, and active NF-κB was
measured. PAR-1AP and PAR-2AP (100 μM) were used as positive con-
trols. Activating solution for gingipains was used as a control. C, Human
gingival fibroblasts with p65- or lamin A/C-specific siRNA for 24 h were
stimulated with gingipains (200 nM). Activating solution for gingipains
was used as a control. After 24 h of stimulation, concentrations of HGF in
the culture supernatants were determined by ELISA. All samples were
assayed in triplicate, and the results are expressed as the mean ± SD.
Asterisks indicate a significant difference from the respective control.
IL-1α, whereas LPS, a well-known virulence factor from Gram-negative bacteria, was completely inactive in this respect (15). It must be noted that LTA specimens by themselves had only marginal activities (15), and muramylpeptidase, a bacterial peptidoglycan whose intracellular receptor was recently revealed to be a nucleotide-binding oligomerization domain 2 (NOD2), had no effect on HGF production by human gingival fibroblasts (A. Uehara and H. Takada, unpublished observation). Therefore, it is noteworthy that HGF was remarkably induced in human gingival fibroblasts by fimbriae and gingipains, both of which were representative virulence factors peculiar to putative periodontopathogenic Porphyromonas gingivalis. In this context, HGF levels in gingival crevicular fluid also increase with periodontitis (55, 56), and the HGF concentration in gingival crevicular fluid from healthy gingival tissue is ~2 ng/ml (55), ~10-fold that in serum from healthy subjects. These data indicate that HGF is actually produced in various tissues, including healthy gingival tissue. Furthermore, clinical data show that various diseases, including inflammation, degeneration, and oncogenesis, cause the augmentation of HGF levels in human body fluids (55, 57–63).

Oral mucosa heals faster with less scar formation than skin. A recent study has revealed that HGF expression by oral mucosa fibroblasts is elevated compared with expression by dermal fibroblasts (64). Because c-Met is reported to be expressed in many epithelial cells, the mitogenic activity of HGF to epithelial cells, including gingival epithelial cells, is known to play a role in wound healing. However, in addition to HGF acting as a mitogen in wound healing, matrix metalloproteinases also play a fundamental role in tissue remodeling. HGF enhances the production of matrix metalloproteinases by keratinocytes derived from skin or oral mucosa (65, 66). HGF also stimulates blood vessel formation and promotes vascularization (67), a later process in wound healing. In this context, it is noteworthy that the expression of vascular endothelial growth factor, a potent angiogenic factor, is also enhanced by HGF (68). Moreover, HGF regulates tight junctions to inhibit the appearance of occluding/ZO-1 in the membrane, thus stimulating cell dissociation and migration (69). These activities of HGF are thought to be involved in wound healing in tissues.

Taking our present results together with previous reports of the activation of PAR-2 on neutrophils (40), PAR-1 and PAR-4 on platelets (41), and PAR-1 and PAR-2 on oral epithelial cells (42) by gingipains, these data establish a new paradigm in microbial pathogenicity; specifically, that some host cell functions may be manipulated by bacterial proteases cleaving the PARs. In the case of P. gingivalis, the immediate advantage of this new pathway for the pathogen may not be clearly apparent. However, uncontrolled PAR activation will certainly contribute to deregulation of the local inflammatory reaction, which can be beneficial for the microbial community in the pathogenic periodontal pocket. In addition to the PARs, several other cell surface receptors, including C5a (CD88) (28), FMLP-R (70), LPS receptor (CD14) (30, 31), αβ1 integrin (71), occludin, and E-cadherin (72), are substrates for Rgp- and/or Kgp-specific gingipains. Therefore, it is conceivable that the modification of molecules on host cell surfaces by bacterial proteases may play an important role in the maintenance of the chronic inflammatory condition associated with periodontitis. A potentially deleterious effect of cell surface receptor cleavage was also shown for metalloproteases from Serratia marcescens, Staphylococcus aureus, Pseudomonas aeruginosa, and Listeria monocytogenes as well as a cysteine protease from Streptococcus pyogenes (73, 74). Taken together, these data firmly establish that proteolytic modification of host cell surfaces by bacterial proteases represents a newly discovered virulence pathway used by some pathogens.

P. gingivalis is the most widely studied periodontopathic bacterium (17). Therefore, HGF induced by gingipains might have a role in some periodontal diseases. In fact, it has been suggested that HGF is responsible for osteoclast formation (75). The HGF receptor, c-Met, is expressed on both osteoclast-like cells and osteoblasts (76). HGF induces shape changes and stimulates the chemotactic migration and proliferation of osteoclasts, and these cells then synthesize and secrete biologically active HGF, suggesting the existence of autocrine and paracrine regulation mechanisms for osteoclasts and osteoblasts, respectively, by HGF. The possible multiple functions of HGF in gingival fibroblasts may be involved in both destructive and reparative phases in periodontal diseases under different physiological and pathological conditions. HGF has been shown to induce blood vessel formation in vitro (77) and in vivo (65) and may be involved in tissue regeneration. At present, studies of HGF in relation to periodontal disease are still in the initial stages, and additional findings are eagerly awaited.

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References


