Dual regulation of interleukin-8 production in human oral epithelial cells upon stimulation with gingipains from *Porphyromonas gingivalis*

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Cysteine proteinases from *Porphyromonas gingivalis*, or gingipains, are considered to be key virulence factors of the bacterium in relation to periodontal diseases. Incubation of human oral epithelial cells with lysine-specific gingipain (Kgp) and high-molecular-mass arginine-specific gingipain (HRgpA) resulted in a decrease in the production of interleukin (IL)-8, but not in the production of other pro-inflammatory cytokines. In contrast, arginine-specific gingipain 2 (RgpB) increased IL-8 production. RNA interference assays demonstrated that Kgp- and HRgpA-mediated downregulation and RgpB-mediated upregulation occurred through protease-activated receptor (PAR)-1 and PAR-2 signalling. Although the RgpB-mediated upregulation of IL-8 production occurred through nuclear factor-kappa B (NF- κ B), the Kgp- and HRgpA-mediated downregulation was not negated in NF- κ B-silenced cells. Both the haemagglutinin and the enzymic domains are required for Kgp and HRgpA to downregulate the production of IL-8 in human oral epithelial cells, and the two domains are thought to co-exist. These results suggest that gingipains preferentially suppress IL-8, resulting in attenuation of the cellular recognition of bacteria, and as a consequence, sustain chronic inflammation.

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INTRODUCTION

Periodontitis is an inflammation of the whole periodontium. The dominant cells in periodontal epithelial tissue are the oral epithelial cells. The barrier function of oral epithelial cells is mainly due to the production of antimicrobial peptides, such as human β -defensins and cathelicidin (Acheson & Luccioli, 2004). Healthy gingival epithelium is characterized by the presence of human β -defensin-2 and a gradient of interleukin (IL)-8 that guides the transmigration of leukocytes (Dixon *et al.*, 2004; Pütsep

et al., 2002). Inflamed gingival epithelium is severely infiltrated with leukocytes around the periodontal pocket (gingival crevice), accompanied by elevated expression of IL-8 (Liu *et al.*, 2001). Chemokines such as IL-8 form the first line of host defence by increasing phagocytosis, bacterial killing, the release of lysosomal enzymes and superoxide anion generation (Weiss, 1989), indicating that this mechanism is of great importance for innate immunity. *Porphyromonas gingivalis* has been implicated not only in severe chronic periodontitis, but also in aggressive periodontitis (Holt & Bramanti, 1991). *P. gingivalis* possesses a number of putative virulence factors, such as LPS, fimbriae and proteinases (Chen *et al.*, 1992). We have studied the virulence activities of two types of trypsin-like cysteine proteinase (Potempa *et al.*, 1995) that cleave specifically at Arg-X (50 and 95 kDa) (Wingrove *et al.*, 1992) and Lys-X (105 kDa) bonds and are referred to as arginine-specific gingipain (Rgp) and lysine-specific gingipain (Kgp), respectively (Pike *et al.*, 1994). The

Abbreviations: FPR-cmk, Phe-Pro-Arg-chloromethyl ketone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRgpA, high-molecular-mass arginine-specific gingipain; IFN- γ , gamma interferon; IL, interleukin; Kgp, lysine-specific gingipain; NF- κ B, nuclear factor-kappa B; PAR, protease-activated receptor; Rgp, arginine-specific gingipain; PAR-1AP, PAR-1 agonist peptide; PAR-2AP, PAR-2 agonist peptide; PAR-3AP, PAR-3 agonist peptide; siRNA, short interfering RNA; z-FKck, benzyl-oxy-carbonyl-Phe-Lys-chloromethyl; TNF- α , tumour necrosis factor alpha.

95 kDa high-molecular-mass arginine-specific gingipain (HRgpA) and Kgp are a complex of the catalytic domain and the haemagglutinin/adhesin domain, and differ from the 50 kDa Rgp (RgpB), which lacks the latter domain. We have shown that gingipains cleave CD14 on human monocytes (Sugawara *et al.*, 2000) and gingival fibroblasts (Tada *et al.*, 2002), and ICAM-1 on human oral epithelial cells (Tada *et al.*, 2003), inhibiting the LPS-elicited defensive response of these cells against this pathogen, and interaction between epithelial cells and leukocytes, respectively, which facilitates *P. gingivalis* in evading the innate immunity. Thus, gingipains are important virulence factors of *P. gingivalis*.

Members of the protease-activated receptor (PAR) family are G protein-coupled receptors (Coughlin, 2000; Déry *et al.*, 1998; O'Brien *et al.*, 2001). There are four members of this family. As PARs are expressed in a wide variety of cell types, it was recently suggested that they may play important roles in pathophysiological processes such as growth, development, inflammation, tissue repair and pain (Coughlin, 2000; Déry *et al.*, 1998; O'Brien *et al.*, 2001). In fact, we demonstrated that neutrophil serine proteinase 3 activates human oral epithelial cells and human gingival fibroblasts via the PAR-2 pathway (Uehara *et al.*, 2003, 2002b). It has been reported that RgpB cleaves and activates PAR-2 on human neutrophils (Lourbakos *et al.*, 1998), induces IL-6 secretion by activating PAR-1 and PAR-2 on human oral epithelial KB cells (Lourbakos *et al.*, 2001a), and causes platelet aggregation via PARs (Lourbakos *et al.*, 2001b). Furthermore, RgpB induces neuropeptide release from dental pulp cells via PAR-2 signalling (Tancharoen *et al.*, 2005). Recently, we revealed that Rgps (HRgpA and RgpB) stimulated the production of hepatocyte growth factor through PAR-1 and PAR-2 in human gingival fibroblasts (Uehara *et al.*, 2005), which may be associated with both inflammatory and reparative processes of periodontal disease. PARs are important molecules that mediate gingipain stimuli to cells. In this study, we analysed the effects of gingipains on the production of cytokines, particularly IL-8, by oral epithelial cells and investigated the possible involvement of PARs in gingipain effects.

METHODS

Reagents. Human natural gamma interferon (IFN- γ) was provided by Hayashibara Biochemical Laboratories. Human recombinant (r)IL-1 α and recombinant tumour necrosis factor alpha (TNF- α) were supplied by Dainippon Pharmaceutical. Phe-Pro-Arg-chloromethyl ketone (FPR-cmk) and benzyloxycarbonyl-Phe-Lys-chloromethyl (z-FKck) were obtained from Bachem Bioscience. PAR-1 agonist peptide (PAR-1AP; SFLLRN), PAR-2 agonist peptide (PAR-2AP; SLIGKV) and PAR-3 agonist peptide (PAR-3AP; TFRGAP) were synthesized by Takara. All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Purification and activation of gingipains. Three forms of gingipain – 95 kDa HRgpA, 50 kDa RgpB and 105 kDa Kgp – were purified from *P. gingivalis* HG66 culture supernatant, as described previously (Pike *et al.*, 1994; Potempa *et al.*, 1998). The purity of each

enzyme was checked by SDS-PAGE. In a 10% Tricine gel, RgpB migrated as a single band with a mobility equivalent to a molecular mass of 48 kDa and homogeneity greater than 95% as determined using laser densitometric scanning of the gel. HRgpA resolved into four major bands and one minor band on SDS-PAGE (Pike *et al.*, 1994). The identity of each protein band was confirmed by N-terminal sequence analysis as being derived from the HRgpA polypeptide. The amount of active enzyme in each purified gingipain was determined by active-site titration using FPR-cmk and z-FKck for the Rgps and Kgp, respectively (Potempa *et al.*, 1997). The concentration of fully activated gingipain with cysteine was calculated from the amount of inhibitor needed for complete inactivation of the proteinases. Therefore, the concentrations of gingipains indicated in this paper are represented as those of active gingipains. The gingipains were activated by diluting to 10 μ M in 0.2 M HEPES (pH 8), 5 mM CaCl₂ and 10 mM cysteine, and incubating at 37 °C for 10 min. The activated gingipains were then diluted with medium or buffer. To block their enzymic activity, the activated gingipains were incubated with the specific inhibitors FPR-cmk and z-FKck for 10 min at room temperature prior to use.

Construction of a strain producing Kgp proteinase without the adhesin domains in a Rgp/Kgp/adhesin-null mutant. The 1.6 kb *EcoRV*–*SmaI* DNA fragment (*cepA* DNA block) of pCS22 (gift from Dr Christine Seers, Cooperative Research Centre for Oral Health Science, School of Dental Science, University of Melbourne, Australia) was inserted into the *SmaI* site of pKD703 (Shoji *et al.*, 2004) with and without the 6.7 kb *XhoI*–*NotI* DNA fragment (*kgp*'–'*rgpB* chimeric gene DNA, blunt-ended) of pKD855 (Sato *et al.*, 2005) to yield plasmids pKD856 (*fimA*::[*kgp*'–'*rgpB cepA*]) and pKD857 (*fimA*::*cepA*), respectively. *ScaI*-linearized pKD856 or pKD857 was introduced into the Rgp/Kgp/adhesin-null mutant KDP153 (Naito *et al.*, 2006), resulting in the transformants KDP154 and KDP160, respectively. KDP160 produces Kgp proteinase without the adhesin domains from the *kgp*'–'*rgpB* chimeric gene.

Collection of supernatants of wild-type *P. gingivalis* and mutant *P. gingivalis* strains and preparation of the adhesin domains rHgp44 and rHbR. *P. gingivalis* 33277 was grown anaerobically to stationary phase in enriched brain heart infusion broth with menadione and haemin without antibiotics. Mutant strains *P. gingivalis* KDP133 (*rgpA rgpB*), *P. gingivalis* KDP136 (*rgpA rgpB kgp*) (Shi *et al.*, 1999), *P. gingivalis* KDP137 (*rgpA kgp hagA*) (Shi *et al.*, 1999), *P. gingivalis* KDP153 (*rgpA rgpB kgp hagA*), KDP160 (*rgpA rgpB kgp hagA fimA*::[*kgp*'–'*rgpB*]) and KDP161 (*rgpA rgpB kgp hagA fimA*) (Table 1) were grown anaerobically to stationary phase in enriched brain heart infusion broth with menadione, haemin and erythromycin (10 μ g ml^{–1}). After 2 days of culture, supernatants were obtained by centrifugation at 10 000 g for 20 min at 4 °C and

Table 1. Phenotype of protein fractions prepared from culture supernatants of wild-type and various mutant *P. gingivalis* strains

Strain	<i>rgpA</i>	<i>rgpB</i>	<i>kgp</i>	<i>hagA</i>
33277	+	+	+	+
KDP133	–	–	+	–
KDP137	–	+	–	+
KDP136	–	–	–	+
KDP153	–	–	–	–
KDP160	–	–	+	–
KDP161	–	–	–	–

then precipitated with 75 % saturated ammonium sulphate at 4 °C for 1 h. The precipitate was collected by centrifugation at 10 000 *g* for 20 min at 4 °C and then dialysed three times against 0.05 % Brij35 (Sigma-Aldrich) in 10 mM sodium phosphate buffer (pH 7) at 4 °C. The procedure used to prepare supernatants of *P. gingivalis* was based on the purification technique for gingipain as described in detail previously (Kadowaki *et al.*, 1994). Purification of the rHgp44 and rHBR (Hgp15) adhesin proteins was conducted as described previously (Naito *et al.*, 2006; Nakayama *et al.*, 1998).

Cells and cell culture. Human oral epithelial cell line HSC-2 (Momose *et al.*, 1989), established from a squamous cell carcinoma, was obtained from the Cancer Cell Repository, Institute of Development, Ageing and Cancer, Tohoku University, Japan. HSC-2 cells were grown in RPMI 1640 (Nissui Seiyaku) with 10 % heat-inactivated fetal calf serum (Life Technologies) with a change of medium every 3 days. To avoid the possibility of trypsinization affecting the amounts of PAR and other surface markers, we used cell dissociation solution (Sigma-Aldrich), which contains no protein and allows the dislodging of cells without the use of enzymes; thus, cellular proteins are preserved without enzymic modification or the adsorption of foreign proteins.

ELISA of cytokines. Cells (1×10^4 in 200 μ l) were incubated with or without stimulant in RPMI 1640 with 10 % fetal calf serum for 24 h in 96-well, flat-bottomed plates (Falcon). Culture supernatants were collected and levels of IL-8 were determined using an ELISA kit (BD Pharmingen). The concentrations of cytokines in the supernatants were determined using the LS-PLATE 2004 data analysis program (Wako Pure Chemical Industries).

RT-PCR assay. Total cellular RNA was obtained using Isogen (Nippon Gene), and was reverse transcribed using random hexamer primers and avian myeloblastosis virus transcriptase XL. The primers used for PCR were as follows: IL-8, 5'-GATTGAGAGTGGACCACT-3' and 5'-TCTCCCGTGAATATCTAGG-3'; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TGAAGGTCTGGAGTCAACGGATTGGT-3' and 5'-TGAAGGTCTGGAGTCAACGGATTGGT-3', generating fragments of 422 and 286 bp, respectively. Cycling conditions were 25 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min. Amplified samples were visualized on 2 % agarose gels stained with ethidium bromide and photographed under UV light.

RNA interference. Transfection for targeting endogenous PAR-1, PAR-2 and nuclear factor-kappa B (NF- κ B) subunit p65 was carried out using Lipofectamine 2000 (Invitrogen) and short interfering RNA (siRNA) (final concentration 200 nM), according to the manufacturer's instructions. siRNA of PAR-1, PAR-2 and NF- κ B p65, and anti-NF- κ B p65 antibody (mouse IgG2a), were purchased from Santa Cruz Biotechnology.

NF- κ B activity. Activated NF- κ B was measured with an NF- κ B assay kit specific for the p65 subunit according to the manufacturer's instructions (Active Motif). Briefly, samples of whole-cell extracts (1–10 μ g protein per well) were added to 96-well plates coated with an oligonucleotide containing the NF- κ B consensus site (5'-GGGACTTCC-3') and incubated for 1 h at room temperature with mild agitation. After three washes, NF- κ B p65 antibody was added for 1 h without agitation, followed by horseradish peroxidase-conjugated anti-mouse IgG1. Colorimetric reactions were developed and stopped and the absorbance measured at 450 nm. The specificity of binding was also examined using an oligonucleotide containing a wild-type or mutated NF- κ B consensus binding site.

Data analysis. Statistical significances were determined using ANOVA with the Bonferroni or Dunnett method.

RESULTS AND DISCUSSION

The oral epithelium is directly exposed to periodontal bacteria, and their products may play an important role in host defence mechanisms against pathogens. To investigate the effects of gingipains (HRgpA, RgpB and Kgp) on the secretion of IL-8 from human oral epithelial cells, HSC-2 cells were incubated with gingipains. We found a dual effect of gingipains on IL-8 secretion of oral epithelial cells: a decrease by complex forms carrying haemagglutinin/adhesin domains (Kgp and HRgpA) and an increase by a form lacking the haemagglutinin domains (RgpB) (Fig. 1). Spontaneous IL-8 secretion was decreased by Kgp and HRgpA at concentrations of 20–200 nM after 24 h incubation, but, in contrast, was increased by RgpB at 200 nM (Fig. 1a). The downregulation by Kgp and HRgpA and the upregulation by RgpB were also significant after a 12 h incubation, reaching a maximum at 24 h and continuing until 48 h (Fig. 1b). RNA was then extracted from oral epithelial cells stimulated with gingipains and RT-PCR was performed to define the level of IL-8 mRNA. IL-8 mRNA was expressed in untreated cells and the expression of IL-8 mRNA was significantly downregulated by Kgp and HRgpA, but not by RgpB (Fig. 1c). It should be noted that Kgp and HRgpA did not cause a decrease in the production of other pro-inflammatory cytokines (IL-1 α , IL-6, monocyte chemoattractant protein-1 and TNF- α) (data not shown). In contrast, PAR-1AP and PAR-2AP, but not PAR-3AP, clearly upregulated IL-8 production (Fig. 1d), which was consistent with results of our previous studies (Uehara *et al.*, 2002b, 2004). We also examined whether Kgp and/or HRgpA was capable of inhibiting enhanced IL-8 production induced by pro-inflammatory cytokines in oral epithelial cells. As shown in Fig. 2, Kgp and/or HRgpA clearly inhibited the production of IL-8 induced by IFN- γ , IL-1 α or TNF- α in oral epithelial cells almost to baseline level. The findings suggested outstanding downregulatory effects of gingipains on IL-8 secretion. It must be noted here that similar results to those obtained using HSC-2 cells in Figs 1 and 2, and in additional experiments, were also obtained from primary oral epithelial cells and two other oral epithelial cell lines (data not shown).

We then examined whether the Kgp- and HRgpA-mediated downregulation and RgpB-mediated upregulation of IL-8 production in oral epithelial cells were due to the enzymic activities of gingipains. It has been reported that the enzymic activity of Rgp and Kgp are inhibited specifically by FPR-cmk and z-FKck, respectively (Potempa *et al.*, 1997). FPR-cmk, a specific inhibitor of Rgps, almost completely negated the RgpB-induced upregulation and HRgpA-induced downregulation (Fig. 3). z-FKck, a specific inhibitor of Kgp, also clearly inhibited the Kgp-mediated downregulation of IL-8 production (Fig. 3). These results indicated that RgpB-mediated upregulation, Kgp-mediated downregulation and HRgpA-mediated downregulation of IL-8 production were dependent on their enzymic activities.

Fig. 1. Dual regulation of IL-8 secretion by gingipains and PAR agonist peptides in human oral epithelial cells in culture. (a, b, d) HSC-2 cells were stimulated with gingipains (Kgp, HRgpA or RgpB) at the concentrations indicated for 24 h (a), with 200 nM gingipains for the periods indicated (b), or with PAR agonist peptides (200 μ M) for 24 h (d), in triplicate at 37 °C. Activating solution for gingipains (a, b) was used as a control. IL-8 levels in the culture supernatants were determined by ELISA and expressed as means $\pm$ SD. *, $P < 0.01$ compared with medium alone. (c) HSC-2 cells were stimulated without or with 200 nM gingipain (Kgp, HRgpA or RgpB) for 6 h, and the expression of IL-8 and GAPDH mRNA was analysed by RT-PCR.

We next examined whether downregulation and/or upregulation of IL-8 production also occurred through PAR family members. As demonstrated previously, human oral epithelial cells constitutively express mRNAs and cell-surface proteins of PAR-1, PAR-2 and PAR-3 but not PAR-4 (Uehara *et al.*, 2002b). To block PAR expression on oral epithelial cells, we used siRNAs for PARs. As shown previously (Uehara *et al.*, 2005), transfection of human oral epithelial cells with PAR-1-, PAR-2- or PAR-3-specific siRNA results in an approximately 80 % decrease in the

Fig. 2. Downregulation of IL-8 secretion induced by inflammatory cytokines in human oral epithelial cells in culture. HSC-2 cells were incubated with or without IFN- γ (1000 IU ml $^{-1}$), IL-1 α (10 ng ml $^{-1}$) or TNF- α (10 ng ml $^{-1}$) for 24 h. After three washes with PBS, cells were incubated for 24 h in the presence or absence of gingipain (200 nM) at 37 °C. IL-8 concentration was determined by ELISA. * and # indicate that values differ significantly compared with the respective controls.

level of PAR-1, PAR-2 or PAR-3 mRNA, but not GAPDH mRNA, in cells cultured for 24–72 h. In both PAR-1- and PAR-2-siRNA transfected cells, Kgp- and HRgpA-mediated downregulation was significantly eliminated and RgpB-mediated upregulation was also suppressed, but the effect of PAR-3-siRNA transfection was not significant (Fig. 4). The decrease in IL-8 production by Kgp and HRgpA in epithelial cells is believed to be the first report that gingipains inhibit cellular function via PARs.

In our previous study (Uehara *et al.*, 2005), the Rgp-induced production of hepatocyte growth factor occurred via NF- κ B downstream of PAR signalling. As shown in Fig. 5(a), Kgp, HRgpA and RgpB significantly increased

Fig. 3. Effect of specific inhibitors for gingipains, cytochalasin B and cycloheximide, on gingipain-mediated regulation of IL-8 secretion. Gingipains were pre-treated with FPR-cmk (10 μ M) or z-FKck (100 μ M) for 15 min at 37 °C before use. HSC-2 cells were stimulated with or without gingipains for 24 h. Activating solution for gingipains was used as a control. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as means $\pm$ SD. *, $P < 0.01$ compared with the respective control.

Fig. 4. Involvement of PAR-1 and PAR-2 in gingipain-mediated downregulation of IL-8 production in human oral epithelial cells. HSC-2 cells were transfected with PAR-1-, PAR-2- or PAR-3-specific-siRNA for 24 h, and incubated for an additional 24 h in the presence or absence of gingipains (200 nM) at 37 °C. PAR-1AP, PAR-2AP and PAR-3AP were used as reference stimulants. Activating solution for gingipains was used as a control. IL-8 concentrations were determined by ELISA. *, $P < 0.01$ compared with the respective control.

active NF- κ B in human oral epithelial cells. Therefore, we examined the possible involvement of NF- κ B in the regulation of IL-8 by gingipains using siRNA targeting p65, which is a component of NF- κ B. NF- κ B p65 protein levels determined by flow cytometry were reduced by approximately 80 % using specific siRNA in HSC-2 cells up to 72 h (Fig. 5b). Upregulation of IL-8 production by RgpB was significantly inhibited in p65-silenced oral epithelial cells (Fig. 5c). However, downregulation of IL-8 production by Kgp and HRgpA was not inhibited in p65-silenced cells.

Kgp and HRgpA, but not RgpB, are complexes carrying haemagglutinin/adhesin domains (Nakayama *et al.*, 1995; Okamoto *et al.*, 1996), which may be involved in the IL-8-suppressive effects of Kgp and HRgpA. To investigate this possibility, we utilized protein fractions prepared from the culture supernatants of wild-type *P. gingivalis* 33277, *rgpA*- and *rgpB*-defective mutant *P. gingivalis* KDP133, *rgpA*-, *kgp*- and *hagA*-defective mutant *P. gingivalis* KDP137, *rgpA*-, *rgpB*- and *kgp*-defective mutant *P. gingivalis* KDP136, and a mutant KDP160, which carries only the enzymic domain of Kgp transfected into the Rgp/Kgp/adhesin-defective mutant *P. gingivalis* KDP153. KDP161 was used as a control for KDP160. The protein fraction from wild-type *P. gingivalis*, which contains Kgp and HRgpA, and KDP133, which also contains Kgp, significantly inhibited IL-8 secretion, and the protein fraction from KDP136, which contains only the adhesin domain, slightly inhibited the secretion, whereas the partially purified protein from KDP160, which contains the haemagglutinin domain-defective Kgp, and KDP137,

Fig. 5. NF- κ B is not involved in gingipain-mediated downregulation of IL-8 production in human oral epithelial cells. (a) HSC-2 cells were incubated for 1 h in the presence or absence of gingipains (200 nM) at 37 °C and active NF- κ B was determined by ELISA. Activating solution for gingipains was used as a control. (b) HSC-2 cells were transfected with siRNA for NF- κ B p65. After 0 and 72 h, the cells were stained with anti-NF- κ B p65 antibody at 4 °C for 30 min, followed by FITC-conjugated goat anti-mouse IgG. (c) HSC-2 cells transfected with p65-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, IL-8 concentration in the culture supernatants was determined by ELISA. All samples were assayed in triplicate and the results are expressed as means $\pm$ SD. *, Significant difference compared with the respective control.

which contains RgpB, significantly stimulated IL-8 production (Fig. 6a). These findings suggested that the adhesin domain may be required for the downregulation of IL-8 production by HRgpA and Kgp, although the adhesin domain alone is not sufficient to exert full inhibitory activity. C-terminal adhesin domains (HbR and Hgp44) are responsible for haemagglutination. We examined the effect on HSC-2 cells of rHbR and/or rHgp44 plus RgpB. We did not observe any downregulation of IL-8 production (data not shown). Furthermore, rHbR and rHgp44 marginally but significantly downregulated IL-8 secretion (Fig. 6b). These results suggested that both catalytic

Fig. 6. The haemagglutinin domain is necessary but not sufficient for gingipains to downregulate IL-8 production in human oral epithelial cells. (a) HSC-2 cells were cultured with or without 0.1–1 µg protein fraction ml⁻¹ prepared from culture supernatants of wild-type *P. gingivalis* 33277 and mutant *P. gingivalis* strains KDP133, KDP137, KDP136, KDP153, KDP160 and KDP161 for 24 h in triplicate at 37 °C. IL-8 concentration was determined by ELISA and the results shown as means ± SD. (b) HSC-2 cells were cultured with or without 0.01–10 µg recombinant C-terminal adhesin domains (rHbR and/or rHgp44) ml⁻¹ for 24 h in triplicate at 37 °C. IL-8 concentrations were determined by ELISA and the results shown as means ± SD. *, *P* < 0.01 compared with the respective control.

(enzymic) and haemagglutinin domains exist in the same molecule for Kgp and HrGpA, and exert a powerful downregulatory effect on IL-8 production in human oral epithelial cells.

As gingipains are reported to cleave pro-inflammatory cytokines such as IL-8 (Mikolajczyk-Pawlinska *et al.*, 1998), it may be possible that the enzymic activity of gingipains directly cleaves IL-8. It must be emphasized, however, that in our study clear inhibition of IL-8 mRNA expression by Kgp and HrGpA, but not RgpB, was observed (Fig. 1c). RgpB carrying enzymic activity did not decrease IL-8 production, and mutant KDP137, which carries only the enzymic domain of Kgp, and mutant KDP160, which carries only the enzymic domain of RgpB, also did not decrease IL-8 production (Fig. 6). In addition, we clarified, using RNA interference, that dual regulation of IL-8 production by gingipains involves PAR-1, PAR-2 and the NF-κB signalling pathway (Fig. 4).

Downregulation of IL-8 secretion by gingipains may be a novel mechanism by which *P. gingivalis* evades the host defence system. We demonstrated previously that human oral epithelial cells treated with pro-inflammatory cytokines (IFN-γ, IL-1α or TNF-α) secreted a high level of various pro-inflammatory cytokines, including IL-8, in response to bacterial cell-surface components (Uehara *et al.*, 2002a), although naive human oral epithelial cells in culture did not show enhanced production of pro-inflammatory cytokines upon stimulation with these stimuli (Uehara *et al.*, 2001). Therefore, in the presence of gingipains, oral epithelial cells might be totally devoid of IL-8 production, even upon stimulation with bacterial components. It must be noted that *P. gingivalis* LPS, another putative virulence factor, is suggested to evade recognition by the host via Toll-like receptor 4 (Ogawa *et al.*, 2007). Considering all of these findings, *P. gingivalis* could inhabit periodontal tissues by evading host defence mechanisms and, as a consequence, sustain chronic inflammation.

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REFERENCES

- Acheson, D. W. K. & Luccioli, S. (2004). Microbial–gut interactions in health and disease. Mucosal immune responses. *Best Pract Res Clin Gastroenterol* **18**, 387–404.
- Chen, Z., Potempa, J., Polanowski, A., Wikstrom, M. & Travis, J. (1992). Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from *Porphyromonas gingivalis*. *J Biol Chem* **267**, 18896–18901.
- Coughlin, S. R. (2000). Thrombin signalling and protease-activated receptors. *Nature* **407**, 258–264.
- Déry, O., Corvera, C. U., Steinhoff, M. & Bunnett, N. W. (1998). Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol* **274**, C1429–C1452.
- Dixon, D. R., Bainbridge, B. W. & Darveau, R. P. (2004). Modulation of the innate immune response within the periodontium. *Periodontol* **2000** **35**, 53–74.
- Holt, S. C. & Bramanti, T. E. (1991). Factors in virulence expression and their role in periodontal disease pathogenesis. *Crit Rev Oral Biol Med* **2**, 177–281.
- Kadowaki, T., Yoneda, M., Okamoto, K., Maeda, K. & Yamamoto, K. (1994). Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. *J Biol Chem* **269**, 21371–21378.
- Liu, R. K., Cao, C. F., Meng, H. X. & Gao, Y. (2001). Polymorphonuclear neutrophils and their mediators in gingival

- tissues from generalized aggressive periodontitis. *J Periodontol* 72, 1545–1553.
- Lourbakos, A., Chinni, C., Thompson, P., Potempa, J., Travis, J., Mackie, E. J. & Pike, R. N. (1998). Cleavage and activation of proteinase-activated receptor-2 on human neutrophils by gingipain-R from *Porphyromonas gingivalis*. *FEBS Lett* 435, 45–48.
- Lourbakos, A., Potempa, J., Travis, J., D'Andrea, M. R., Andrade-Gordon, P., Santulli, R., Mackie, E. J. & Pike, R. N. (2001a). Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. *Infect Immun* 69, 5121–5130.
- Lourbakos, A., Yuan, Y., Jenkins, A. L., Travis, J., Andrade-Gordon, P., Santulli, R., Potempa, J. & Pike, R. N. (2001b). Activation of protease-activated receptors by gingipains from *Porphyromonas gingivalis* leads to platelet aggregation: a new trait in microbial pathogenicity. *Blood* 97, 3790–3797.
- Mikolajczyk-Pawlinska, J., Travis, J. & Potempa, J. (1998). Modulation of interleukin-8 activity by gingipains from *Porphyromonas gingivalis*: implications for pathogenicity of periodontal disease. *FEBS Lett* 440, 282–286.
- Momose, F., Araida, T., Negishi, A., Ichijo, H., Shioda, S. & Sasaki, S. (1989). Variant sublines with different metastatic potentials selected in nude mice from human oral squamous cell carcinomas. *J Oral Pathol Med* 18, 391–395.
- Naito, M., Sakai, E., Shi, Y., Ideguchi, H., Shoji, M., Ohara, N., Naito, M., Yamamoto, K. & Nakayama, K. (2006). *Porphyromonas gingivalis*-induced platelet aggregation in plasma depends on Hgp44 adhesin but not Rgp proteinase. *Mol Microbiol* 59, 152–167.
- Nakayama, K., Kadowaki, T., Okamoto, K. & Yamamoto, K. (1995). Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis*. Evidence for significant contribution of Arg-gingipain to virulence. *J Biol Chem* 270, 23619–23626.
- Nakayama, K., Ratnayake, D. B., Tsukuba, T., Kadowaki, T., Yamamoto, K. & Fujimura, S. (1998). Haemoglobin receptor protein is intragenically encoded by the cysteine proteinase-encoding genes and the haemagglutinin-encoding gene of *Porphyromonas gingivalis*. *Mol Microbiol* 27, 51–61.
- O'Brien, P. J., Molino, M., Kahn, M. & Brass, L. F. (2001). Protease activated receptors: theme and variations. *Oncogene* 20, 1570–1581.
- Ogawa, T., Asai, Y., Makimura, Y. & Tamai, R. (2007). Chemical structure and immunobiological activity of *Porphyromonas gingivalis* lipid A. *Front Biosci* 12, 3795–3812.
- Okamoto, K., Kadowaki, T., Nakayama, K. & Yamamoto, K. (1996). Cloning and sequencing of the gene encoding a novel lysine-specific cysteine proteinase (Lys-gingipain) in *Porphyromonas gingivalis*: structural relationship with the arginine-specific cysteine proteinase (Arg-gingipain). *J Biochem (Tokyo)* 120, 398–406.
- Pike, R., McGraw, W., Potempa, J. & Travis, J. (1994). Lysine- and arginine-specific proteinases from *Porphyromonas gingivalis*. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. *J Biol Chem* 269, 406–411.
- Potempa, J., Pike, R. & Travis, J. (1995). The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. *Infect Immun* 63, 1176–1182.
- Potempa, J., Pike, R. & Travis, J. (1997). Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. *Biol Chem* 378, 223–230.
- Potempa, J., Mikolajczyk-Pawlinska, J., Brassell, D., Nelson, D., Thøgersen, I. B., Enghild, J. J. & Travis, J. (1998). Comparative properties of two cysteine proteinases (gingipains R), the products of two related but individual genes of *Porphyromonas gingivalis*. *J Biol Chem* 273, 21648–21657.
- Pütsep, K., Carlsson, G., Boman, H. G. & Andersson, M. (2002). Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet* 360, 1144–1149.
- Sato, K., Sakai, E., Veith, P. D., Shoji, M., Kikuchi, Y., Yukitake, H., Ohara, N., Naito, M., Okamoto, K. & other authors (2005). Identification of a new membrane-associated protein that influences transport/maturation of gingipains and adhesins of *Porphyromonas gingivalis*. *J Biol Chem* 280, 8668–8677.
- Shi, Y., Ratnayake, D. B., Okamoto, K., Abe, N., Yamamoto, K. & Nakayama, K. (1999). Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. *J Biol Chem* 274, 17955–17960.
- Shoji, M., Naito, M., Yukitake, H., Sato, K., Sakai, E., Ohara, N. & Nakayama, K. (2004). The major structural components of two cell surface filaments of *Porphyromonas gingivalis* are matured through lipoprotein precursors. *Mol Microbiol* 52, 1513–1525.
- Sugawara, S., Nemoto, E., Tada, H., Miyake, K., Imamura, T. & Takada, H. (2000). Proteolysis of human monocyte CD14 by cysteine proteinases (gingipains) from *Porphyromonas gingivalis* leading to lipopolysaccharide hyporesponsiveness. *J Immunol* 165, 411–418.
- Tada, H., Sugawara, S., Nemoto, E., Takahashi, N., Imamura, T., Potempa, J., Travis, J., Shimauchi, H. & Takada, H. (2002). Proteolysis of CD14 on human gingival fibroblasts by arginine-specific cysteine proteinases from *Porphyromonas gingivalis* leading to down-regulation of lipopolysaccharide-induced interleukin-8 production. *Infect Immun* 70, 3304–3307.
- Tada, H., Sugawara, S., Nemoto, E., Imamura, T., Potempa, J., Travis, J., Shimauchi, H. & Takada, H. (2003). Proteolysis of ICAM-1 on human oral epithelial cells by gingipains. *J Dent Res* 82, 796–801.
- Tancharoen, S., Sarker, K. P., Imamura, T., Biswas, K. K., Matsushita, K., Tatsuyama, S., Travis, J., Potempa, J., Torii, M. & Maruyama, I. (2005). Neuropeptide release from dental pulp cells by RgpB via proteinase-activated receptor-2 signaling. *J Immunol* 174, 5796–5804.
- Uehara, A., Sugawara, S., Tamai, R. & Takada, H. (2001). Contrasting responses of human gingival and colonic epithelial cells to lipopolysaccharides, lipoteichoic acids and peptidoglycans in the presence of soluble CD14. *Med Microbiol Immunol* 189, 185–192.
- Uehara, A., Sugawara, S. & Takada, H. (2002a). Priming of human oral epithelial cells by interferon- γ to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans. *J Med Microbiol* 51, 626–634.
- Uehara, A., Sugawara, S., Muramoto, K. & Takada, H. (2002b). Activation of human oral epithelial cells by neutrophil proteinase 3 through protease-activated receptor-2. *J Immunol* 169, 4594–4603.
- Uehara, A., Muramoto, K., Takada, H. & Sugawara, S. (2003). Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. *J Immunol* 170, 5690–5696.
- Uehara, A., Sugawara, S., Sasano, T., Takada, H. & Sugawara, S. (2004). Proinflammatory cytokines induce proteinase 3 as membrane-bound and secretory forms in human oral epithelial cells and antibodies to proteinase 3 activate the cells through protease-activated receptor-2. *J Immunol* 173, 4179–4189.
- Uehara, A., Muramoto, K., Imamura, T., Nakayama, K., Potempa, J., Travis, J., Sugawara, S. & Takada, H. (2005). Arginine-specific gingipains from *Porphyromonas gingivalis* stimulate production of hepatocyte growth factor (scatter factor) through protease-activated

receptors in human gingival fibroblasts in culture. *J Immunol* 175, 6076–6084.

Weiss, S. J. (1989). Tissue destruction by neutrophils. *N Engl J Med* 320, 365–376.

Wingrove, J. A., DiScipio, R. G., Chen, Z., Potempa, J., Travis, J. & Hugli, T. E. (1992). Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas* (*Bacteroides*) *gingivalis*. *J Biol Chem* 267, 18902–18907.