Cleavage of protease-activated receptors on an immortalized oral epithelial cell line by *Porphyromonas gingivalis* gingipains

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Porphyromonas gingivalis activates protease-activated receptors (PARs) on oral keratinocytes, resulting in downstream signalling for an innate immune response. Activation depends on *P. gingivalis* gingipains, but could be confounded by lipopolysaccharide signalling through Toll-like receptors. We therefore hypothesized that *P. gingivalis* cleaves oral keratinocyte PARs in an Arg- (Rgp) or Lys- (Kgp) gingipain-specific manner to upregulate pro-inflammatory cytokines. Immortalized human oral keratinocytes (TERT-2) were incubated with wild-type *P. gingivalis* (ATCC 33277) or strains from a panel of isogenic gingipain deletion mutants: Kgp-deficient (KDP 129); Rgp-deficient (KDP 133); or Kgp- and Rgp-deficient (KDP 136). After incubation with *P. gingivalis*, keratinocytes were probed with specific antibodies against the N-terminus of PAR-1 and PAR-2. Using flow cytometry and immunofluorescence, receptor cleavage was marked by loss of specific antibody binding to the respective PARs. TERT-2 cells constitutively expressed high levels of PAR-1 and PAR-2, and lower levels of PAR-3. *P. gingivalis* ATCC 33277 cleaved PAR-1 and PAR-2 in a dose-dependent manner, while the receptors were unaffected by the protease-negative double mutant (KDP 136) at all m.o.i. tested. The single Kgp-negative mutant preferentially cleaved PAR-1, whereas the Rgp-negative mutant cleaved PAR-2. Wild-type or Kgp-negative mutant cleavage of PAR-1 upregulated expression of IL-1 α , IL-1 β , IL-6 and TNF- α ; the Rgp-negative mutant did not modulate these cytokines. Selective cleavage of PAR-1 on oral epithelial cells by *P. gingivalis* Rgp therefore upregulates expression of pro-inflammatory cytokines.

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INTRODUCTION

Porphyromonas gingivalis is a Gram-negative anaerobe present in subgingival plaque and widely associated with periodontitis in adults (Chen *et al.*, 2001; Nonnenmacher *et al.*, 2004, 2005). Periodontal diseases and inflammation are associated with specific *P. gingivalis* virulence factors (Tatakis & Kumar, 2005), including fimbriae (Miura *et al.*, 2005; Weinberg *et al.*, 1997), lipopolysaccharides (LPS) (Wang *et al.*, 2002) and gingipains (Kadowaki *et al.*, 1994, 2003; Pathirana *et al.*, 2007a; Potempa *et al.*, 2000; Yoneda *et al.*, 1990). Gingipains are cysteine proteases produced by *P. gingivalis* (Kadowaki *et al.*, 1994; Potempa *et al.*, 2000). Two types of gingipains have been described: arginine-

specific (Arg-gingipains or Rgp) and lysine-specific (Lys-gingipains or Kgp). Rgp can be found as three variants: a 50 kDa RgpB, a 50 kDa RgpA_{cat} and a 95 kDa HRgpA (Potempa *et al.*, 2000). In a mouse model, Kgp and RgpB, but not RgpA, contribute to periodontal disease (Pathirana *et al.*, 2007a), findings that were confirmed by the use of novel gingipain inhibitors (Kadowaki & Yamamoto, 2003; Kadowaki *et al.*, 2004).

On eukaryotic cell membranes, gingipains cleave the N-terminal domains to activate the protease-activated receptors (PARs), members of the seven-transmembrane superfamily of cell-surface G-protein-coupled receptors (reviewed by Coughlin, 2000; Ossovskaya & Bunnett, 2004). Four PARs are currently described: PAR-1, PAR-2, PAR-3 and PAR-4. Hence, *P. gingivalis* gingipains cleave and activate PARs on neutrophils (Lourbakos *et al.*, 1998) and platelets (Lourbakos *et al.*, 2001b), and Rgp activation of PAR-2 has been linked to inflammation and induction

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Abbreviations: Kgp, lysine-specific gingipain(s); PAR, protease-activated receptor; Rgp, arginine-specific gingipain(s); TLR, Toll-like receptor.

of alveolar bone loss in periodontitis (Holzhausen *et al.*, 2005, 2006). Consistent with these findings, PAR-2-deficient mice lose less alveolar bone than wild-type mice after challenge with *P. gingivalis* supernatants, which appears to be caused by Rgp signalling through PAR-2 (Holzhausen *et al.*, 2006). In KB cells (a HeLa-like carcinoma cell line), however, *P. gingivalis* Rgp appears to activate PAR-1 and PAR-2 and release IL-6, a potent stimulator of osteoclast differentiation and bone resorption (Lourbakos *et al.*, 2001a). Hence, the PAR–gingipain specificity leading to release of pro-inflammatory cytokines is unclear, in part complicated by the presence of *P. gingivalis* LPS with concomitant activation through Toll-like receptors (TLRs) also leading to expression of pro-inflammatory cytokines (Chen *et al.*, 2008; Diya *et al.*, 2008; Eskan *et al.*, 2008).

In the present study, we determined the *P. gingivalis* gingipain cleavage specificity for PARs on oral keratinocytes. By using an immortalized oral epithelial cell line, TERT-2, we have shown that PAR-1 and PAR-2 are differentially cleaved by *P. gingivalis* Rgp and Kgp to upregulate expression of pro-inflammatory cytokines.

METHODS

Cell cultures. OKF6/TERT-2 (TERT-2), an immortalized oral epithelial cell line provided by J. Rheinwald (Harvard Medical School, Cambridge, MA) (Dickson *et al.*, 2000), was used to model primary gingival epithelial cells. TERT-2 cells were grown in 5% CO₂ at 37 °C in keratinocyte serum-free and calcium-free medium (Invitrogen Life Technologies) supplemented with 0.4 mM CaCl₂, 25 µg ml⁻¹ bovine pituitary extract and 0.2 ng ml⁻¹ epidermal growth factor, as previously reported (Giacaman *et al.*, 2007). Culture medium was changed every 3 days and cells were subcultured when they reached approximately 60% confluence.

Bacterial strains and culture conditions. *P. gingivalis* strain ATCC 33277 and a panel of isogenic deletion mutants that fail to express Kgp (KDP 129; Δ kgp) (Okamoto *et al.*, 1998), Rgp (KDP 133; Δ rgpA Δ rgpB) (Nakayama *et al.*, 1995) or both gingipains (KDP 136; Δ kgp Δ rgpA Δ rgpB) (Shi *et al.*, 1999) (kindly provided by K. Nakayama, Nagasaki University, Nagasaki, Japan) were used. *P. gingivalis* strains were grown anaerobically in a Coy chamber (85% N₂, 5% CO₂ and 10% H₂) at 37 °C on Todd–Hewitt agar plates (Difco) or in Todd–Hewitt broth. Agar and broth were supplemented with 5 µg haemin ml⁻¹ and 1 µg menadione ml⁻¹ (both Sigma-Aldrich). Agar plates were also supplemented with 5% (v/v) defibrinated sheep blood. Bacteria were grown to early stationary-phase (OD₆₂₀ ~ 0.8–1.0). Spent bacterial broth with early stationary phase *P. gingivalis* cells containing secreted and cell-wall-associated gingipains (Potempa *et al.*, 2000) was added to keratinocyte cultures at 37 °C at an m.o.i. (bacteria to keratinocytes) of 10:1, 100:1 or 1000:1. In preparation, keratinocytes at 60% confluence were washed with Dulbecco's PBS (DPBS) (Mediatech) and the medium was replaced with pre-warmed serum-free MEM (Mediatech). In the presence of inoculated *P. gingivalis*, TERT-2 cells were incubated at 37 °C in 5% CO₂ for 1 h. Control TERT-2 cells were inoculated with fresh sterile *P. gingivalis* growth medium and maintained under the same conditions. For some control experiments, TERT-2 cells were incubated for 30 min in serum-free MEM (Mediatech) at 37 °C in 5% CO₂ with either 100 nM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich) or 100 nM Δ -thrombin (MP

Biomedicals). After incubation with *P. gingivalis*, sterile control medium, TPCK-trypsin or thrombin, keratinocytes were washed three times with DPBS and processed for the appropriate analysis as below.

Flow cytometry. After incubation with *P. gingivalis* or controls, TERT-2 cells were washed three times with DPBS and detached from the flasks or wells using 0.2% (w/v) EDTA and gentle scraping. Since trypsin activates PARs (Lourbakos *et al.*, 2001a), this enzyme was not used to detach the cells. Cells were counted, fixed for 10 min at 4 °C in 4% (w/v) paraformaldehyde and reacted with optimized dilutions of primary antibodies against PAR-1, -2 and -3. The antibodies used were murine monoclonal antibodies against PAR-1 (ATAP) (Santa Cruz Biotechnology) or PAR-2 (Zymed), or rabbit polyclonal antibody against PAR-3 (Santa Cruz Biotechnology). Primary antibodies and cells were incubated for 30 min at 4 °C, washed three times with PBS supplemented with 2% (v/v) FBS, and washed cells were then incubated in the dark for 30 min with a 1:100 dilution of goat anti-murine IgG secondary antibody conjugated with phycoerythrin (Jackson ImmunoResearch Laboratories) to detect PAR-1 or PAR-2, or with goat anti-rabbit IgG secondary antibody conjugated with FITC (Sigma) for PAR-3. Murine monoclonal antibodies IgG1 and IgG2a (BD Biosciences and BD Pharmingen) were used as isotype controls for PAR-1 and PAR-2, respectively. For PAR-3 detection, rabbit polyclonal IgG was used as isotype control. Fluorescent signals were monitored with a FACScan (BD Biosciences) using CellQuest software. Each antibody was analysed and reactivity was confirmed in at least three independent experiments.

Immunofluorescence microscopy. TERT-2 cells (2×10^5) were grown as described above and seeded on coverslips in 12-well plates. Cells (60% confluent) were incubated with *P. gingivalis* for 1 h at various m.o.i. After incubation with *P. gingivalis*, cells were washed three times with DPBS, fixed in 4% (w/v) paraformaldehyde, and blocked with 3% (w/v) BSA for 30 min at room temperature to minimize non-specific antibody binding. To detect PARs, TERT-2 cells were incubated with 80 µl of a 1:100 dilution of murine monoclonal antibody against PAR-1 (ATAP) or PAR-2 (SAM-11) (Santa Cruz Biotechnology) for 1 h in the dark. Murine IgG1 and IgG2a antibodies were used as isotype controls for PAR-1 and PAR-2, respectively. Cells were washed five times with DPBS and then incubated in the dark for one additional hour with Alexa Fluor 568-conjugated goat anti-murine IgG Antibody (Molecular Probes) and 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) at 1:2000 and 1:300 dilutions, respectively. Coverslips were washed five times, mounted on glass slides, and cells were observed using a Nikon Eclipse E800 fluorescence microscope. All experiments were performed in triplicate.

Quantitative PCR. Since incubation with *P. gingivalis* for longer than 6 h causes cells to detach from the flasks (Nisapakultorn *et al.*, 2001), keratinocytes were incubated in TERT medium with *P. gingivalis* for 3 h, as previously described (Giacaman *et al.*, 2007), washed five times with DPBS and then incubation continued for the times indicated. At times up to 48 h after initial exposure to *P. gingivalis*, TERT-2 cells were harvested. Total RNA was extracted using an Rneasy Plus Mini kit (Qiagen) according to the manufacturer's instructions, quantified using a 2100 Bioanalyser (Agilent Technologies), and 500 ng was used as template to synthesize cDNA using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was then diluted 1:5 with RNase/DNase-free water and 1 µl (5 ng) was used as template in the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). Quantitative PCR was performed on each sample in triplicate using an ABI7900 HT (Applied Biosystems). Data were analysed using SDS 2.1 software (Applied Biosystems), normalizing all genes to human β -actin (SuperArray Bioscience). RT² qPCR primers (SuperArray Bioscience) were used to detect specific mRNAs for the cytokines IL-1 α , IL-1 β , IL-6, TNF- α , IL-8 and IFN- γ . At each time

point, expression of the selected mRNAs in cells incubated with *P. gingivalis* was calculated relative to the housekeeping gene β -actin (ΔC_t) for each sample, and then expressed relative to untreated cells at the same time point using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

Statistical analyses. Statistical analyses were performed by Student's *t* test for paired values using GraphPad software (GraphPad Software). Differences were considered significant at $P < 0.05$.

RESULTS

Expression of PAR-1, PAR-2 and PAR-3 on oral epithelial cells

To learn whether TERT-2 cells constitutively express surface PARs, resting, untreated keratinocytes were analysed by flow cytometry for PAR-1, PAR-2 and PAR-3. TERT-2 cells expressed surface PAR-1, -2 and -3 (Fig. 1). PAR-1 and PAR-2 were more frequently expressed at detectable levels than PAR-3 ($P < 0.001$).

Thrombin and trypsin effects on PAR cleavage

Since thrombin and trypsin cleave PARs (Hansen *et al.*, 2004), we treated TERT-2 cells to confirm cleavage of PAR-1 and PAR-2. Compared to levels on untreated cells, PAR-1 was significantly cleaved (60 % reduction) by thrombin (Fig. 2A, E), but PAR-2 was unaffected (Fig. 2B, I). In contrast, trypsin cleaved both PAR-1 (Fig. 2A, F) and PAR-2 (Fig. 2B, J) to barely detectable levels. Although PAR-3 was detected at low levels (Fig. 1), thrombin and trypsin apparently did not cleave this receptor (data not shown). For all subsequent experiments, thrombin and trypsin were used as positive controls for PAR-1, and PAR-1 and PAR-2 cleavage, respectively.

Fig. 1. PAR expression in oral keratinocytes. TERT-2 cells, grown as described in Methods, were analysed by flow cytometry to assess constitutive expression of surface PAR-1, PAR-2 and PAR-3. Cells were seeded in flasks and harvested without trypsin as described. Bars represent the mean $\pm$ SEM of five independent experiments. ** $P < 0.001$, relative to PAR-1.

P. gingivalis cleavage of PAR-1 and PAR-2 receptors on oral epithelial cells

TERT-2 cells were incubated for 1 h with *P. gingivalis* ATCC 33277 in spent medium at various m.o.i. to verify cleavage of PARs. *P. gingivalis* cleaved PAR-1 (Fig. 3A) and PAR-2 (Fig. 3B) in a dose-dependent manner. PAR-1 (Fig. 3A) was cleaved more effectively than PAR-2 at each m.o.i. tested (Fig. 3B) and PAR-2 cleavage was only detectable at m.o.i. greater than 100:1 (Fig. 3B). At m.o.i. 100:1 and 1000:1, *P. gingivalis* reduced PAR-1 signals to levels comparable to trypsin treatment and similar to untreated cells incubated with the isotype control antibody (Fig. 3A). Cleavage of the PARs by *P. gingivalis* was confirmed by immunofluorescence microscopy (Fig. 3C–L).

Effect of gingipains on PAR cleavage

To determine whether gingipains produced by intact cells of *P. gingivalis* were responsible for PAR cleavage, keratinocytes were incubated with an isogenic mutant strain, KDP 136, which fails to express either gingipain. Unlike the wild-type, KDP 136 was unable to cleave either PAR-1 or PAR-2 at any m.o.i. tested up to 1000:1 (data not shown). To investigate the role of each *P. gingivalis* cysteine protease on PAR cleavage, TERT-2 cells were incubated with a Lys- (KDP 129) or Arg-gingipain-deletion mutant (KDP 133), or with the wild-type ATCC 33277. *P. gingivalis* KDP 129 cleaved PAR-1 (Fig. 4A), but not PAR-2 (Fig. 4B), suggesting that cleavage of PAR-1 was Arg-gingipain specific. When oral keratinocytes were incubated with KDP 133, which does not express the Arg-gingipains (Fig. 4C, D), the percentage of PAR-1 positive cells was only slightly reduced (Fig. 4C) whereas the percentage of PAR-2 positive cells was reduced to levels similar to the wild-type strain (Fig. 4D). This suggested that PAR-2 cleavage was largely Lys-gingipain-dependent. Since PAR-2 was significantly cleaved by the wild-type, cleaved only moderately by the Rgp-deficient mutant and was unaffected by the double mutant, the two proteases are suggested to act synergistically to cleave PAR-2.

Gingipain-mediated regulation of pro-inflammatory cytokines

To determine which gingipain upregulates expression of pro-inflammatory cytokines, oral keratinocytes were incubated with *P. gingivalis* ATCC 33277 or the gingipain mutant strains. IL-1 α , IL-1 β , IL-6, TNF- α , IL-8 and IFN- γ expression was analysed by real-time PCR. By 6 h, *P. gingivalis* induced significant increases in the expression of pro-inflammatory cytokines IL-1 α , IL-1 β , IL-6 and TNF- α (Fig. 5A–D), but not IL-8 and IFN- γ (data not shown), by TERT-2 cells. Furthermore, upregulation of IL-1 β , IL-6, IL-1 α and TNF- α appeared to be gingipain-dependent. While Δ Kgp (KDP 129) and the wild-type induced expression of a similar cytokine profile, the Δ Rgp (KDP 133) and the double mutant KDP 136 were unable to upregulate the

Fig. 2. PAR cleavage by thrombin and trypsin. Oral keratinocytes were incubated with medium, thrombin or trypsin (100 nM) for 30 min at 37 °C in 5 % CO₂. For flow cytometry (A, B), cells were detached from the vessels using 0.2 % (w/v) EDTA, fixed, blocked, stained with isotype IgG, PAR-1 or PAR-2 antibodies, and analysed to determine protease cleavage of PAR-1 (A) and PAR-2 (B) on the cell surface. For immunofluorescence (C–J), cells were incubated with either fresh medium (C, D, G, H) or proteases (E, F, I, J), fixed, blocked and stained with isotype control IgG (C and G) or antibodies against PAR-1 (D–F) or PAR-2 (H–J), as described. Bars represent the mean $\pm$ SEM of three independent experiments. ***P* < 0.001 when compared to 'No treatment'. For both assays, IgG1 (A, C) and IgG2a (B, G) isotype antibodies were used as negative controls at the same concentration as antibodies used to detect PAR. Scale bars, 8 μ m. Blue, DAPI; red, Alexa Fluor 568-conjugated IgG. Images are representative of two independent experiments.

pro-inflammatory cytokines. These findings suggest that Arg-gingipains selectively cleave PAR-1 to upregulate pro-inflammatory cytokines in oral keratinocytes.

DISCUSSION

Oral keratinocytes express pro-inflammatory cytokines in response to indigenous pathogens such as *P. gingivalis*. As we have reported (Giacaman *et al.*, 2007), *P. gingivalis* Rgp upregulates CCR5 through PAR-1 and -2 signalling. Hence, the PARs enhance the responses of keratinocytes to cytokines. In addition to selective upregulation of chemo-

kine receptors, we now provide strong evidence that PAR signalling also increases the expression of pro-inflammatory cytokines, exclusive of LPS-mediated responses through TLRs (reviewed by Potempa & Pike, 2009).

Virulence of *P. gingivalis* against oral epithelial cells has often been characterized in KB cells (Eick *et al.*, 2006; Madianos *et al.*, 1996; Pathirana *et al.*, 2007b). Although KB cells were originally derived from an oral epidermal carcinoma, further analysis demonstrated HeLa markers in the chromosomes. KB cells are now considered HeLa contaminants (American Type Culture Collection, ATCC). In our studies we used an immortalized oral keratinocyte

Fig. 3. *P. gingivalis* cleavage of PAR receptors. TERT-2 cells were incubated for 1 h at 37 °C in 5% CO₂ with fresh medium or with *P. gingivalis* ATCC 33277 (WT) in spent culture medium at m.o.i. 10:1, 100:1 or 1000:1, washed and prepared for flow cytometry (A, B) or immunofluorescence (C–L), as described in Methods. For flow cytometry, cells were detached from the vessels using 0.2% (w/v) EDTA, fixed, blocked, stained and analysed to determine protease cleavage of PAR-1 (A) and PAR-2 (B) on the cell surface. PAR-1 and PAR-2 cleavage was visualized by immunofluorescence. *P. gingivalis*-exposed (E–G and J–L) and unexposed (C, D, H, I) cells were fixed, blocked and stained on coverslips for PAR-1 (D–G) or PAR-2 (I–L), as described. Bars represent the mean $\pm$ SEM of three independent experiments. * P < 0.05; ** P < 0.001, when compared to 'No treatment'. For both assays, IgG1 (A, C) and IgG2a (B, H) isotype antibodies were used as negative controls. Scale bars, 12 μ m. Blue, DAPI; red, Alexa Fluor 568-conjugated IgG. Fluorescence images are representative of two independent experiments.

cell line, TERT-2, obtained from oral mucosa (Dickson *et al.*, 2000). Immortalized cells behave more consistently *in vitro* than primary cells from different donors. Unlike HeLa cells, TERT-2 cells retain the phenotype of the original oral keratinocytes. Using indirect approaches, *P. gingivalis* gingipains have been shown to activate PARs. Downstream signalling responses reported include calcium mobilization (Holzhausen *et al.*, 2006; Loubakos *et al.*, 1998) and cytokine production (Holzhausen *et al.*, 2006; Loubakos *et al.*, 2001a; Tancharoen *et al.*, 2005). We now provide direct evidence of PAR receptor cleavage by arg- and lys-gingipains using flow cytometry and immunofluorescence. Binding of specific antibody indicates that the extracellular domain of a PAR is intact and loss of binding shows that the domain has been cleaved. Consistent with previous reports (Loubakos *et al.*, 2001a), resting,

untreated keratinocytes show high expression of PAR-1 and PAR-2 and weak expression of PAR-3 (Fig. 1). PAR-4 has not been described in the oral epithelium (Loubakos *et al.*, 2001a). After incubation with thrombin, trypsin or *P. gingivalis* cells, ectodomain-specific PAR-1 and -2 antibodies fail to bind, indicating receptor cleavage.

Like the wild-type strain, the gingipain-deletion mutants are assumed to express LPS, although we cannot rule out alternative variants. Since the TLRs are intact and functional on the oral keratinocytes (Giacaman *et al.*, 2007), differences in keratinocyte responses to gingipain mutants are unlikely to be attributable to differential signalling through the TLRs. In our system, *P. gingivalis* cleaves PAR-1 (Fig. 3A and E–G) and PAR-2 (Fig. 3B and J–L). A *P. gingivalis* mutant strain that expresses neither

Fig. 4. Arg- and Lys-gingipain specific PAR proteolysis. Oral keratinocytes were incubated for 1 h with fresh medium (Isotype and No treatment), at m.o.i. 1000 : 1 of *P. gingivalis* ATCC 33277 or at m.o.i. 10 : 1, 100 : 1 or 1000 : 1 of *P. gingivalis* KDP 129 (A, B) or *P. gingivalis* KDP 133 (C, D), deletion mutants for Lys- and Arg-gingipains, respectively. Cells were analysed for PAR-1 (A, C) and PAR-2 (B, D) using flow cytometry as described in the legend of Fig. 3. Wild-type *P. gingivalis* and isotype antibodies, as described in Fig. 3, were used as positive and negative controls, respectively. Graphs depict mean $\pm$ SEM of three independent experiments. * P <0.05; ** P <0.001, when compared to 'No treatment'.

gingipain fails to cleave PARs on oral keratinocytes, suggesting cleavage by gingipains. Since *P. gingivalis* Rgp and Kgp cannot individually simulate PAR-2 cleavage induced by the wild-type (Fig. 4B, D), the two gingipains might act synergistically to cleave and activate PAR-2. Yet, *P. gingivalis* KDP 129 (Δ Kgp) alone cleaves PAR-1 at an m.o.i. similar to *P. gingivalis* ATCC 33277 (Fig. 4A). Rgp is therefore suggested to cleave PAR-1 more effectively than PAR-2. Kgp has also been reported to be abnormally processed in an Rgp mutant (Kadowaki *et al.*, 1998). Furthermore, only Rgp possesses the specificity to cleave the PAR-2-tethered ligand between Arg³⁴ and Ser³⁵ (Uehara *et al.*, 2005). Abnormal processing of Kgp could explain why neither the Rgp mutant nor the double gingipain mutant could simulate the PAR-2 cleavage activity of the wild-type strain. Alternatively, the cleavage of PAR-2 could result from digestion by either of the gingipains acting synergistically or with other *P. gingivalis* proteases. Activation and downstream signalling may depend on either Kgp or Rgp, which may explain the attenuated effect of the mutant strains on PAR-2 cleavage.

How selective cleavage of PAR-1 or -2 contributes to differential downstream signalling may be less clear. Although PAR-2 appears relatively insensitive to cleavage

by Rgp (Fig. 4B), Rgp activation and downstream signalling appears to occur through PAR-2 (Holzhausen *et al.*, 2005, 2006; Lourbakos *et al.*, 1998, 2001a; Tanchoen *et al.*, 2005). Hence, the PARs may interact functionally, resulting in pathway cross-talk at some level of signalling, since activation of PAR-1 can co-activate PAR-2 (Ossovskaia & Bunnett, 2004). In primary endothelial cells, specific PAR-1 antagonists fail to completely suppress PAR-1 activation by thrombin. When PAR-2 is also blocked, however, PAR-1 signalling by thrombin is totally abrogated, suggesting transactivation of PAR-2 by cleaved PAR-1 (O'Brien *et al.*, 2000). Conditions in these experiments were similar to our studies. Thus, functional interactions are likely to occur between PAR-1 and PAR-2 signalling pathways.

The release of pro-inflammatory cytokines by keratinocytes represents an innate immune response to pathogens and their virulence factors. Consistent with previous reports (Lourbakos *et al.*, 2001a; Sandros *et al.*, 2000; Uehara *et al.*, 2008a), we show here that the *P. gingivalis* virulence factor Rgp, but not Kgp, selectively induces the expression of pro-inflammatory cytokine mRNA by oral epithelial cells. Cytokine regulation was only verified at the mRNA level. Further studies appear necessary to also show regulation at

Fig. 5. Induction of pro-inflammatory cytokine mRNAs by *P. gingivalis* and gingipain mutants. TERT-2 cells were incubated at m.o.i. 100:1 with *P. gingivalis* ATCC 33277, KDP 129, KDP 133 or KDP 136 in spent bacterial culture medium for 3 h, washed and maintained in fresh medium for the indicated total times. At indicated times, cells were harvested and RNA extracted to perform real-time PCR for IL-1 α (A), IL-1 β (B), IL-6 (C) and TNF- α (D). β -Actin was used as a housekeeping gene. Graphs show expression of each molecule relative to uninfected TERT-2 cells harvested at the same indicated time. * $P < 0.05$, when compared to time 0.

the protein level. Indeed, the gingipains appear to activate PAR-1 to selectively signal the secretion of certain cytokines. Although IL-6 was specifically induced by Rgp, IL-8 was unaffected by either gingipain. A recent report (Uehara *et al.*, 2008b) using the human oral carcinoma cell line HSC-2 suggests that IL-8 is differentially regulated by the purified gingipains. In HSC-2 cells, IL-8 appears to be downregulated by Kgp and HRgpA, while Rgp seems to be upregulated (Uehara *et al.*, 2008b). The immortalized keratinocytes in our study originated from the normal mucosa of the floor of the mouth. Differences in IL-8 responses could be explained by phenotypic differences in the cells but may also reflect differences in the concentrations of specific gingipains associated with freshly harvested *P. gingivalis* and when used as purified proteins in optimized conditions.

The target cell phenotype and co-expressed virulence factors can affect the apparent selectivity of gingipains in upregulating cytokines. For example, *P. gingivalis* Rgp upregulates IL-8 expression in gingival fibroblasts (Oido-Mori *et al.*, 2001), but not in oral epithelial cells as we show here. Furthermore, *P. gingivalis* gingipains are generally co-presented with LPS, which can synergize or simulate PAR-mediated transcriptional responses in oral keratinocytes

(Giacaman *et al.*, 2007). Since all of the *P. gingivalis* mutants used in our study express LPS, differential regulation of pro-inflammatory cytokines argues in favour of gingipain-associated cytokine induction signalled by selective PAR cleavage, rather than from synergistic LPS-mediated activation of TLRs. We cannot, however, rule out that the LPS on the gingipain-mutant strains and the resulting responses in the oral keratinocytes may be altered, given that LPS-gingipain complex formation may mask functional domains of LPS (Takii *et al.*, 2005).

In this complex environment, *P. gingivalis* gingipains have been implicated in the pathogenesis of periodontal disease. *P. gingivalis*-containing dental plaque resides proximal to sulcular gingival keratinocytes, suggesting that activation of PARs and upregulation of pro-inflammatory cytokines could contribute to acute and chronic inflammation in periodontitis. Locally released cytokines can also circulate in the blood (Nagatomo *et al.*, 2007), and *P. gingivalis* gingipains have been associated with platelet aggregation (Lourbakos *et al.*, 2001b), a key event in thrombosis. In mice, systemic inflammation is promoted by gingipain activation of PAR-2 (Holzhausen *et al.*, 2005, 2006). *P. gingivalis* invasion and systemic dissemination of gingipains could therefore link periodontal disease and cardiovascular disease.

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