then precipitated with 75% saturated ammonium sulphate at 4 °C for 1 h. The precipitate was collected by centrifugation at 10000 *g* for 20 min at 4 °C and then dialysed three times against 0.05% Brij35 (Sigma-Aldrich) in 10 nM 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 µl) 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′-GATTGAGAGTGGACC-ACACT-3′ and 5′-TCTCCCGTGCAATATCTAGG-3′; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGAAG-GTCGGAGTCAACGGATTTGGT-3′ and 5′-TGAAGGTCGGAGT-CAACGGATTTGGT-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-κB) 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-κB p65, and anti-NF-κB p65 antibody (mouse IgG2a), were purchased from Santa Cruz Biotechnology.

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 protein per well) were added to 96-well plates coated with an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) and incubated for 1 h at room temperature with mild agitation. After three washes, NF-κB 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-κB consensus binding site.

Data analysis. Statistical significances were determined using ANOVA with the Bonferrori 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-1α, 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-1α 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.

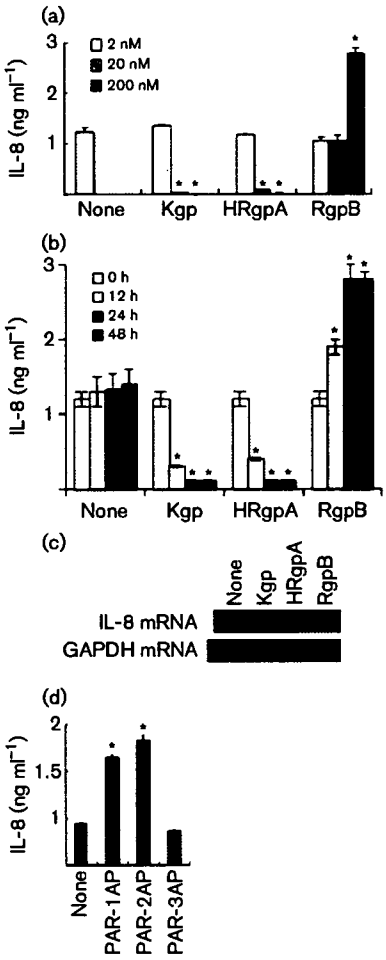

Fig. 1. Dual regulation of IL-8 secretion by gingipains and PAR agonist peptides in human oral epithelial cells in culture. (a, b, d) HSC-2 cells were stimulated with gingipains (Kgp, HRgpA or RgpB) at the concentrations indicated for 24 h (a), with 200 nM gingipains for the periods indicated (b), or with PAR agonist peptides (200 μM) 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 ± 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

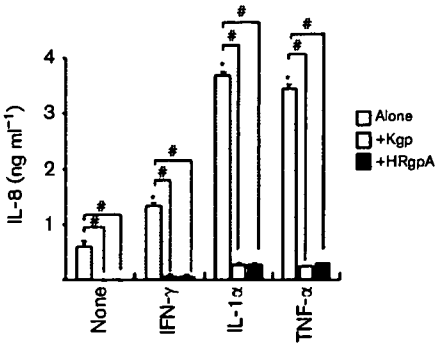

Fig. 2. Downregulation of IL-8 secretion induced by inflammatory cytokines in human oral epithelial cells in culture. HSC-2 cells were incubated with or without IFN-γ (1000 IU ml^{-1}), IL-1α (10 ng ml^{-1}) or TNF-α (10 ng ml^{-1}) for 24 h. After three washes with PBS, cells were incubated for 24 h in the presence or absence of gingipain (200 nM) at 37 °C. IL-8concentration was determined by ELISA. * and #, 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-κB downstream of PAR signalling. As shown in Fig. 5(a), Kgp, HRgpA and RgpB significantly increased

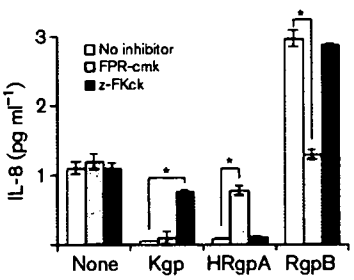

Fig. 3. Effect of specific inhibitors for gingipains, cytochalasin B and cycloheximide on gingipain-mediated regulation of IL-8 secretion. Gingipains were pre-treated with FPR-cmk (10 μM) or z-FKck (100 μM) for 15 min at 37 °C before use. HSC-2 cells were stimulated with or without gingipains for 24 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 ± SD *, $P<0.01$ compared with the respective control.

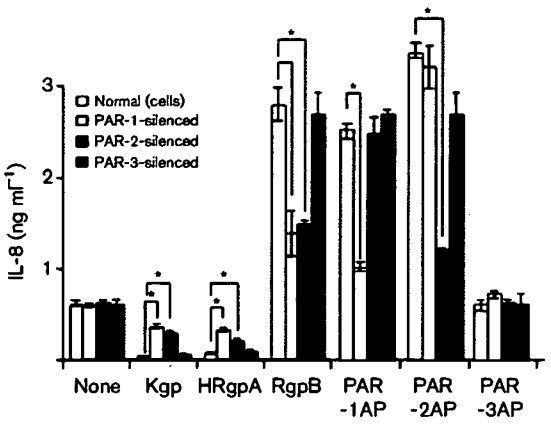

Fig. 4. Involvement of PAR-1 and PAR-2 in gingipain-mediated downregulation of IL-8 production in human oral epithelial cells. HSC-2 cells were transfected with PAR-1-, PAR-2- or PAR-3-specific-siRNA for 24 h and incubated for an additional 24 h in presence or absence of gingipains (200 nM) at 37 °C. 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.

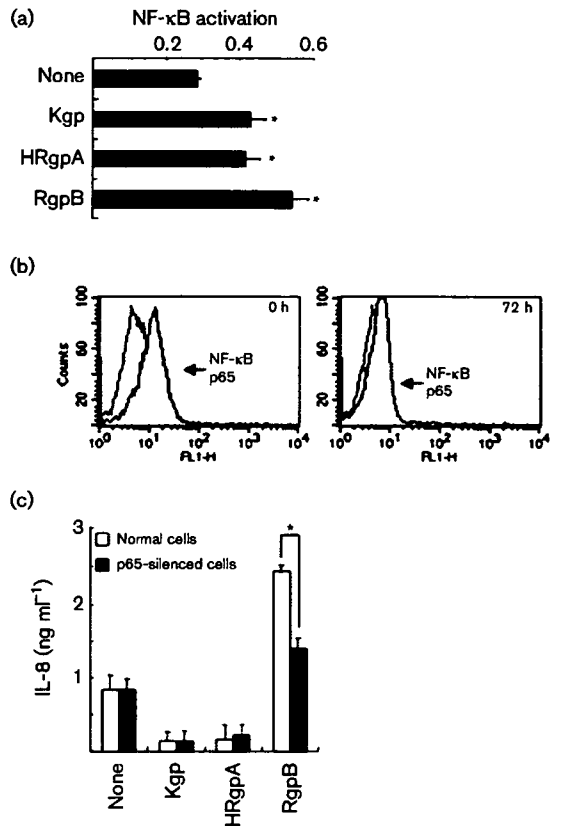

Fig. 5. NF-κB is not involved in gingipain-mediated downregulation of IL-8 production in human oral epithelial cells. (a) HSC-2 cells were incubated for 1 h in the presence or absence of gingipains (200 nM) at 37 °C and active NF-κB was determined by ELISA. Activating solution for gingipains was used as a control. (b) HSC-2 cells were transfected with siRNA for NF-κB p65. After 0 and 72 h, the cells were stained with anti-NF-κB 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 ± SD. *, Significant difference compared with the respective control.

active NF-κB in human oral epithelial cells. Therefore, we examined the possible involvement of NF-κB in the regulation of IL-8 by gingipains using siRNA targeting p65, which is a component of NF-κB. NF-κB 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, *rgp*-, *rgp*- 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, 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

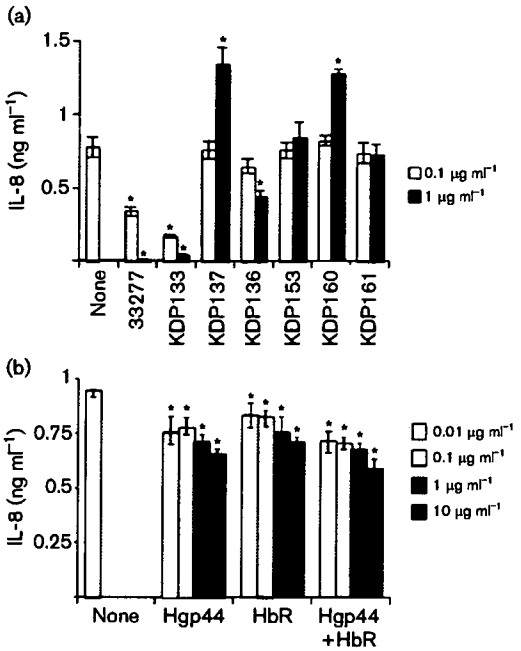

Fig. 6. The haemagglutinin domain is necessary but not sufficient for gingipains to downregulate IL-8 production in human oral epithelial cells. (a) HSC-2 cells were cultured with or without 0.1–1 μg protein fraction ml^{-1} prepared from culture supernatants of wild-type *P. gingivalis* 33277 and mutant *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.

suggested that both catalytic (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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Gingipains from *Porphyromonas gingivalis* synergistically induce the production of proinflammatory cytokines through protease-activated receptors with Toll-like receptor and NOD1/2 ligands in human monocytic cells

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Summary

Gingipains (HRgpA, RgpB and Kgp) are cysteine proteinases and virulence factors of *Porphyromonas gingivalis*, the major causative bacterium of periodontal disease. To study synergistic effects of gingipains and signalling via Toll-like receptors (TLRs) and NOD1/2, we investigated effects of a gingipain on the secretion of proinflammatory cytokines from monocytic THP-1 cells in the presence of pathogen-associated molecular patterns (PAMPs). Gingipains stimulated interleukin (IL)-8's secretion from THP-1 cells, which was completely inhibited by proteinase inhibitors of gingipain and increased in the presence of PAMPs. Synergistic effects of gingipains and PAMPs were also seen in the secretion of IL-6 and MCP-1 and reduced to about 50% the secretion of IL-8 from THP-1 cells treated with siRNA targeting either protease-activated receptor (PAR)-1, -2 or -3. PAR agonist peptides mimicked the synergistic effects of gingipains with PAMPs. These results indicate that gingipains stimulate the secretion of cytokines from monocytic cells through the activation of PARs with synergistic effects by PAMPs. This is the first report of synergism of signalling via PARs, and TLRs or NOD1/2. The host defence system against *P. gingivalis* may be triggered through the activation of PARs by gingipains and augmented by PAMPs from this pathogen via TLRs or NOD1/2.

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Introduction

Periodontal disease is chronic gingival inflammation and causes periodontal tissue destruction, loss of alveolar bone, and eventually, tooth loss. *Porphyromonas gingivalis* is the causative pathogen not only for adult periodontitis but also for rapidly progressive periodontitis (Holt and Bramanti, 1991). *P. gingivalis* possesses a number of putative virulence factors, such as lipopolysaccharide (LPS), fimbriae, toxic products of metabolism and proteinases, all of which stimulate host cells to release inflammatory mediators and promote this infectious disease. We have studied virulence activities of two types of cysteine proteinases (Potempa *et al.*, 1995); arginine-specific gingipains (Rgp) of 50 and 95 kDa that cleave peptide bonds specifically at Arg residues (Chen *et al.*, 1992) and a lysine-specific gingipain (Kgp) of 105 kDa that cleaves peptide bonds specifically at Lys residues (Pike *et al.*, 1994). The 95 kDa high molecular mass Rgp (HRgpA) and kgp are complexes of a catalytic domain and a hemagglutinin/adhesion domain, whereas the 50 kDa Rgp (RgpB) lacks the latter domain. Gingipains enhance vascular permeability through activation of the kallikrein/kinin pathway (Imamura *et al.*, 1994; 1995a), disrupt plasma clotting (Scott *et al.*, 1993; Imamura *et al.*, 1995b; 1997), activate components of the complement system (Wingrove *et al.*, 1992) and modify neutrophil functions (Jagels *et al.*, 1996). The conversion of profimbrilin to mature fimbrilin by gingipains is indispensable for the expression of *P. gingivalis* fimbriae (Kadowaki *et al.*, 1998), an important cell surface structure of this bacterium for adhesion, colonization and invasion (Amano, 2007). Furthermore, 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 LPS-elicited defensive responses of these cells to this pathogen and interaction between epithelial

cells and leukocytes, respectively, which help *P. gingivalis* to evade the innate immune responses.

Protease-activated receptors (PARs) are G protein-coupled receptors, characterized by signal transduction triggered through proteolytic cleavage at each N-terminal peptide (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001). PAR-1, PAR-3 and PAR-4 are activated mainly by thrombin, while PAR-2 is activated by trypsin and mast cell tryptase as well as the coagulation factors VIIa and Xa but not by thrombin (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001). We added neutrophil serine proteinase 3 (PR3) as another activator of PAR-2 on human oral epithelial cells and human gingival fibroblasts (Uehara *et al.*, 2002; 2003). PARs are expressed on a wide variety of cell types, and suggested to play important roles in pathophysiological processes, such as growth, development, inflammation, tissue repair and pain (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001). RgpB activated PAR-2 on human neutrophils (Lourbakos *et al.*, 1998), and induced interleukin (IL)-6's secretion by activating PAR-1 and PAR-2 on human oral epithelial KB cells (Lourbakos *et al.*, 2001a) and platelet aggregation via PAR-1 and PAR-4 (Lourbakos *et al.*, 2001b). Furthermore, RgpB induced the release of neuropeptide from dental pulp cells via PAR-2 signalling (Tancharoen *et al.*, 2005). Recently, we revealed that Rgps (HRgpA and RgpB) stimulated production of hepatocyte growth factor (HGF) through PAR-1 and PAR-2 in human gingival fibroblasts (Uehara *et al.*, 2005a), which may be associated with both inflammatory and reparative processes in periodontal tissues. Therefore, PARs are important molecules that mediate gingipains' stimulatory effects on cells.

The innate immune system recognizes microorganisms through a series of pattern recognition receptors that are highly conserved and bind specifically to common motifs, designated pathogen-associated molecular patterns (PAMPs), present in microorganisms but not in eukaryotes. Representative PAMPs are the lipid A moiety of LPS, lipopeptides, peptidoglycans (PGNs), bacterial DNA, viral double-stranded and single-stranded RNA. Several studies have demonstrated that in mammals, these PAMPs are recognized specifically by the respective Toll-like receptor (TLR) (Akira *et al.*, 2006). In addition, the NOD-like receptor (NLR) family were demonstrated to be intracellular receptors for a partial structure of PGN; NOD1 and NOD2 recognize diaminopimelic acid (DAP) containing a peptide moiety and muramyl peptide respectively (Fritz *et al.*, 2006).

We revealed that a combination of chemically synthesized TLR agonists with muramyldipeptide (MDP) or DAP-containing desmuramylpeptides synergistically induced production of IL-8 in a NOD2- or NOD1-dependent manner, respectively, in human monocytic THP-1 cells in culture (Uehara *et al.*, 2005b). Furthermore, we recently reported that anti-PR3 Abs primes human monocytic cells through PAR-2 for TLR- and NOD-dependent enhanced cell activation (Uehara *et al.*, 2007). These results suggest an interaction of signalling triggered PARs and TLRs or NODs. Therefore, we investigated the possible synergistic effect of a PAR agonist peptide (AP) (PAR-1AP, -2AP or -3AP) and a TLR/NLR ligand agonist on the production of IL-8 in human monocytic cells. Then, we examined whether gingipains as PAR agonists exert a synergistic effect in combination with a TLR/NLR agonist to present a novel activity of gingipains.

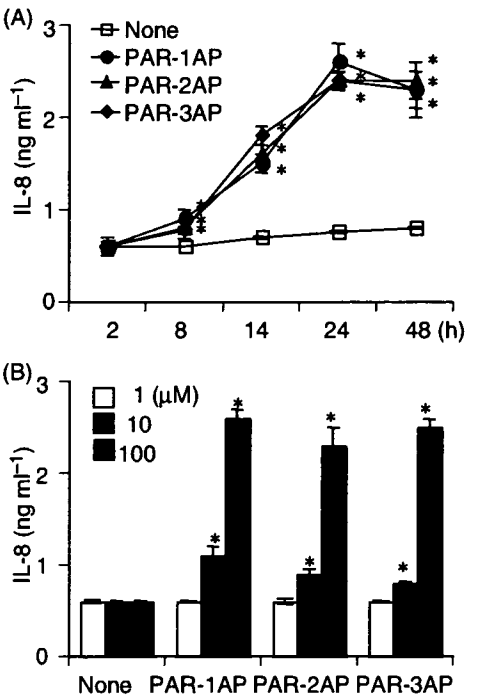

Fig. 1. The time-course and dose response of PAR-AP-induced IL-8 production.
A. THP-1 cells were incubated with 100 μM of PAR-1AP, PAR-2AP or PAR-3AP for a range of different time periods.
B. THP-1 cells were incubated with one of the PAR-APs at various concentrations for 24 h. The concentrations of IL-8 released into supernatants were measured by ELISA and values are the means ± SD for triplicate assays. $^*P < 0.01$ versus the culture medium alone. Results representative of three different experiments are shown.

Results

Increase of IL-8 secretion from cultured human monocytic THP-1 cells by PAR-APs

First, we investigated the effect of PAR signalling (PAR-1, -2 and -3) on the production of IL-8 by THP-1 cells. PAR-APs significantly promoted IL-8's secretion from the cells over an incubation period of 8–24 h, after which there appeared to be a slight decrease (Fig. 1A). PAR-APs

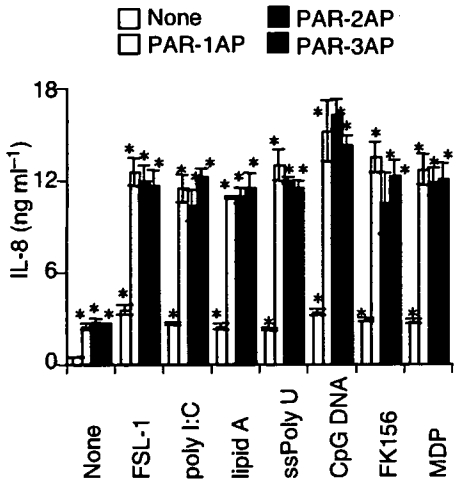

Fig. 2. Synergistic effect of PAR-APs and synthetic TLR and NOD ligands on IL-8 secretion from cultured THP-1 cells. THP-1 cells were stimulated with 100 μM of a PAR-AP in the presence of either FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}) or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8 in the culture supernatants were measured by ELISA, and values are means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone and the respective control respectively. Results representative of three different experiments are shown.

increased the amount of IL-8 secreted in a dose-dependent manner from 10 μM to at least 100 μM (Fig. 1B). The secretion was augmented about fourfold by PAR-APs at 100 μM and the three types of PAR agonists had similar stimulatory effects.

Synergistic effect of PAR-APs and synthetic TLR or NOD ligands on secretion of IL-8 from cultured THP-1 cells

As reported (Uehara *et al.*, 2005b), synthetic PAMPs induced the secretion of IL-8 in THP-1 cells in a dose-dependent manner (data not shown). To elucidate the possible synergistic effects of PAR-APs (PAR-1AP, -2AP and -3AP) and various TLR or NOD agonists, we examined IL-8's secretion from THP-1 cells in the presence of both a PAR-AP and a synthetic PAMP. Clear synergistic effects were observed with all combinations of PAR-APs and PAMPs and the secretion of IL-8 evoked by a PAR-AP increased about four- to fivefold in the presence of any of the PAMPs (Fig. 2).

Increase in secretion of IL-8 caused by gingipains in cultured human monocytic THP-1 cells

We demonstrated that Rgps activate PAR-1 and PAR-2, stimulating the production of hepatocyte growth factor in cultured human gingival fibroblasts (Uehara *et al.*, 2005a). We therefore studied the effects of gingipains (Kgp, HRgpA and RgpB), as PAR agonists, on the secretion of proinflammatory cytokines from THP-1 cells. Gingipains increased production of IL-8 in a time-dependent manner (Fig. 3A). A significant increase in secretion was observed over an incubation period of 14–24 h, and Kgp and HRgpA were more effective than RgpB (Fig. 3A).

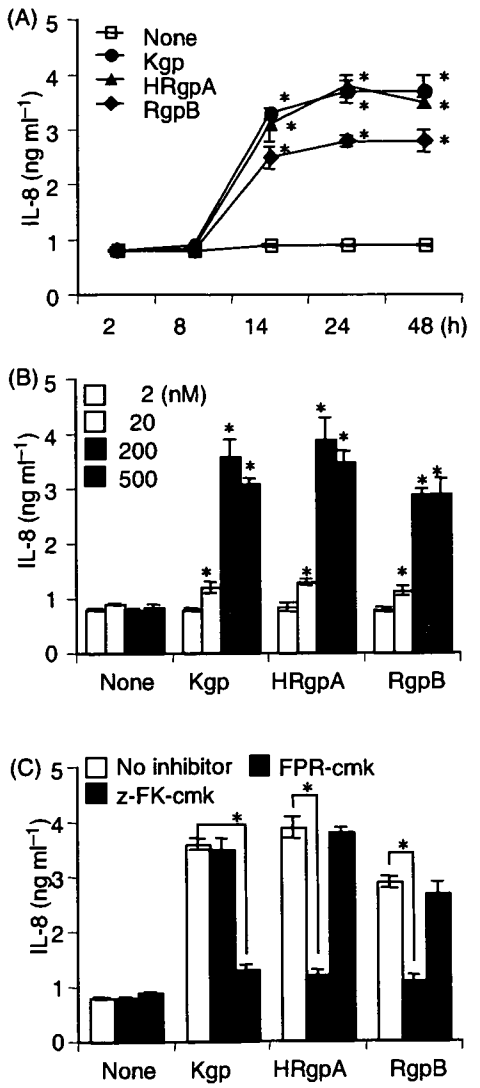

Fig. 3. Effects of gingipains on IL-8 production by THP-1 cells.
A. THP-1 cells were incubated with 200 nM of gingipains for various periods.
B. THP-1 cells were incubated with various concentrations of gingipains for 24 h.
C. THP-1 cells were incubated with gingipains, pretreated with 10 μM of FPR-cmk or z-FK-cmk for 15 min at 37°C, for 24 h. The solution for gingipain activation was used as a control. The concentrations of IL-8 in the supernatant were measured by ELISA and values are the means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone or respective counterpart. Results representative of three different experiments are shown.

Gingipains augmented IL-8's secretion in a dose-dependent manner starting at a gingipain concentration of 20 nM (Fig. 3B). FPR-cmk and z-FK-cmk, inhibitors specific to Rgps and Kgp respectively (Potempa *et al.*, 1997), completely inhibited the gingipain-induced secretion of IL-8, indicating its dependency on the enzymatic activities (Fig. 3C).

Synergistic effect of gingipains and synthetic TLR or NOD ligands on secretion of proinflammatory cytokines from THP-1 cells

Next, we examined the synergistic effects of gingipains and the synthetic PAMPs on cytokine production. Among PAMPs, Poly I:C and CpG DNA induced definite secretion of the three cytokines in the absence of gingipains (Fig. 4). Similar to PAR-APs, the three gingipains exhibited synergistic effects with these PAMPs on IL-8's secretion from THP-1 cells, as was seen for the secretion of IL-6, and monocyte chemoattractant protein (MCP)-1 and HRgpA were most effective in combination with any PAMP, except with FK156 in the secretion (Fig. 4).

Synergistic effect of gingipains and synthetic TLR or NOD ligands on secretion of proinflammatory cytokines from human peripheral blood mononuclear cells (PBMCs)

Next, we examined whether similar synergistic effects of gingipains and synthetic TLR or NOD ligands were observed in human PBMCs. Consistent with the results for THP-1 cells, the gingipains exhibited synergistic effects with these PAMPs on the secretion of IL-8, IL-6 and MCP-1 from PBMCs (Fig. 5).

Involvement of PARs in synergistic effects of gingipains on IL-8 secretion from THP-1 cells

To confirm the involvement of PARs in the synergistic effects of gingipains and PAMPs, TLR agonists or NOD agonists, we used short-interfering RNA (siRNA) to block the expression of PAR-1, -2 or -3. Protein levels of PAR-1, -2 and -3 in the cells treated with the siRNA were suppressed about 80% (Fig. 6A). As shown in Fig. 6B, synergistic effects of gingipains and synthetic PAMPs on the secretion of IL-8 were significantly inhibited to about 50% in all of the PAR-silenced THP-1 cells, but not in Lamin-silenced THP-1 cells, irrespective of PAMPs. These results clearly indicate PAR-1, -2 and -3 to be critical for the synergistic effects of gingipains and TLR or NOD agonists.

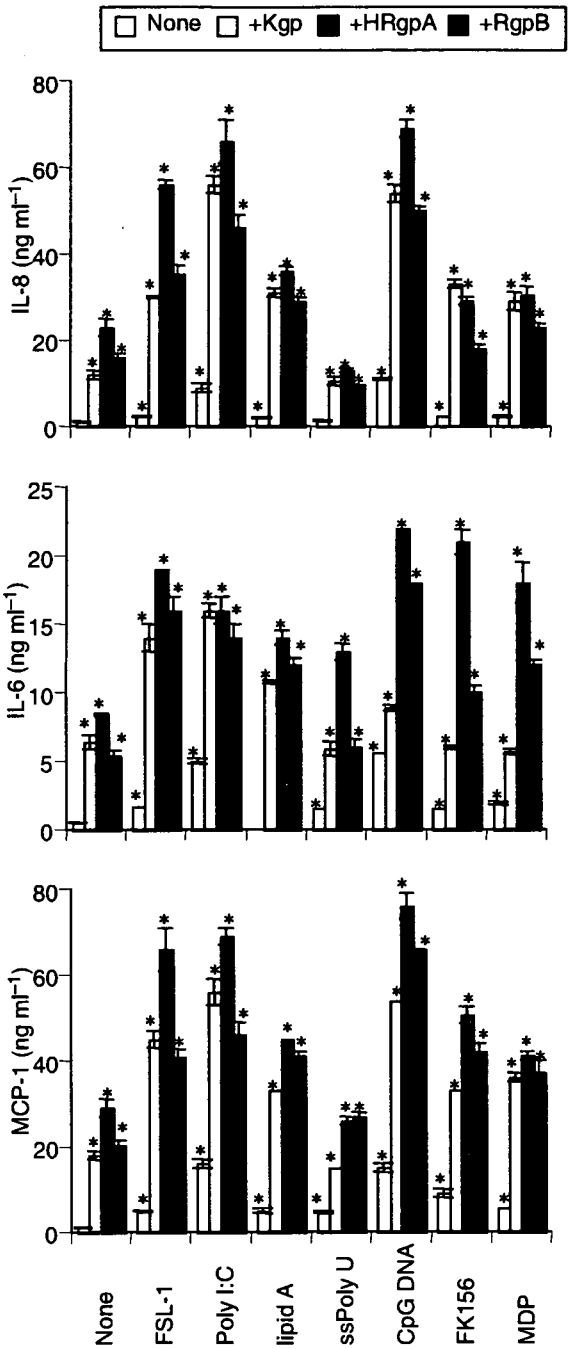

Fig. 4. Synergistic effects of gingipains and synthetic TLR or NOD ligands on secretion of proinflammatory cytokines in cultured THP-1 cells. THP-1 cells were incubated with 200 nM of Kgp, HRgpA, or RgpB in the presence of either of FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}) or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8, IL-6 and MCP-1 in the culture supernatants were measured by ELISA, and values are the means ± SD for triplicate assays. $^*P < 0.01$ versus the culture medium alone and the respective control. Results representative of four different experiments are shown.

Discussion

Porphyromonas gingivalis cysteine proteinases, Rgps and Kgp, synergistically increase the secretion of pro-

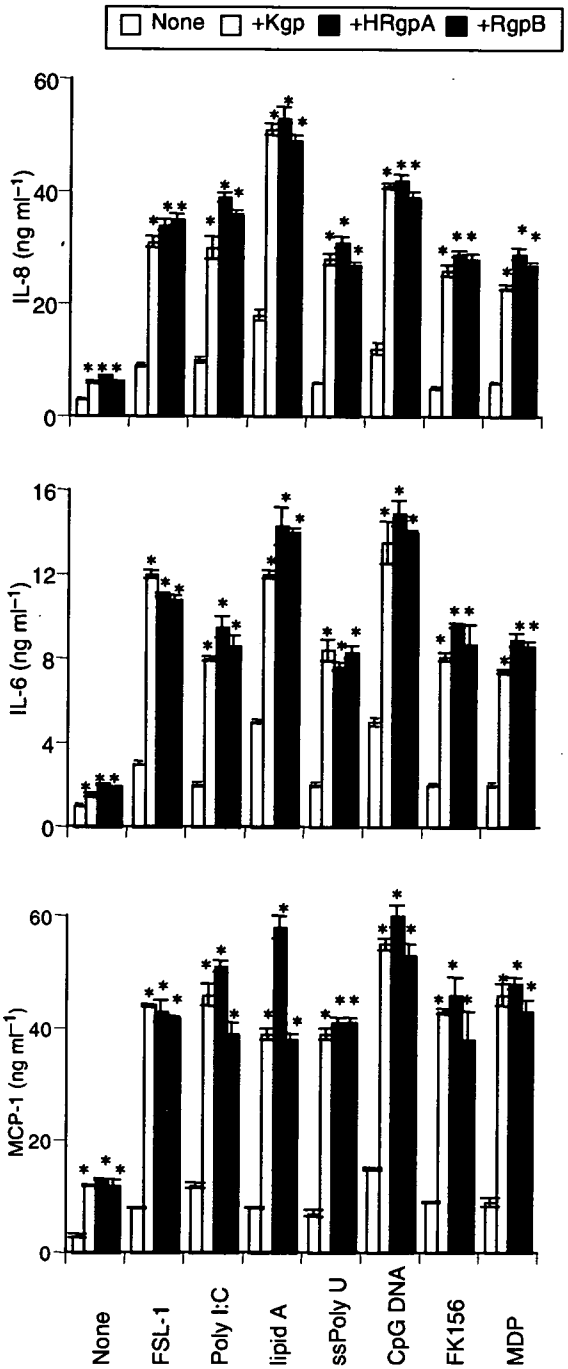

Fig. 5. Synergistic effects of gingipains and, synthetic TLR or NOD ligands on secretion of proinflammatory cytokines in cultured PBMCs. PBMCs were incubated with 200 nM of Kgp, HRgpA, or RgpB in the presence of either of FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}) or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8, IL-6 and MCP-1 in the culture supernatants were measured by ELISA, and values are the means ± SD for triplicate assays. *$P < 0.01$ versus the culture medium alone and the respective control. Results representative of four different experiments are shown.

inflammatory cytokines from human monocytic cells via PAR-1, -2 and -3 in combination with TLR or NOD agonists. This is the first report of synergistic effects of signalling of PARs and TLR or NOD and indicates a link between the PAR system and innate immunity. The involvement of PARs in the synergistic effects were shown by the results that (i) PAR-APs mimicked the effects of gingipains (Fig. 2), (ii) the effects were completely inhibited by treating gingipains with inhibitors specific to each proteinase (Fig. 3C), and (iii) decreasing the expression of PARs by treating cells with siRNA suppressed the effects (Fig. 6B). Activation by gingipains has been shown for PAR-1, -2 and -4 (Lourbakos *et al.*, 2001a,b; Tancharoen *et al.*, 2005; Uehara *et al.*, 2005a) but no report has demonstrated PAR-3's activation. The present results clearly demonstrated that Kgp and Rgps synergistically increased the secretion of proinflammatory cytokines in combination with a TLR or NOD agonist through PAR-1, -2 and -3 (Fig. 6B). It is obvious that the effects of kgp are dependent on the cleavage of PARs by its enzymatic activity (Fig. 3C). PAR-3 is activated by cleavage at the carboxy-terminal side of the Lys residue in the tethered ligand (Déry *et al.*, 1998; Coughlin, 2000; O'Brien *et al.*, 2001), which is consistent with the substrate specificity of Kgp (Pike *et al.*, 1994); however, PAR-1 and PAR-2 require cleavage after the Arg residue and are activated by Rgps. The result that the synergistic effects of gingipains and synthetic PAMPs on IL-8 secretion were inhibited by about 50% in all PAR-silenced THP-1 cells irrespective of the PAMPs indicates an involvement of Rgps and Kgp in the activation of PAR-1 and PAR-2 and PAR-3 respectively (Fig. 6B). Although in Figs 3 and 4 there seems to be some differences in IL-8 production between Kgp, HRgpA and RgpB, in Fig. 5 such differences appear to disappear by a combination of each gingipain with PAMPs. Therefore, the synergism between gingipains and PAMPs varies depending on the combination used, cells used and cytokines secreted. Anyway, the present study shows cross-talk between the PAR system, and TLR or NOD1/2 system, and suggests a new interaction between bacteria and the host defence system. However, previous studies have demonstrated that gingipains would rapidly and efficiently inactivate (Calkins *et al.*, 1998; Baba *et al.*, 2002; Bodet *et al.*, 2005) or be related to decreased extracellular levels of various proinflammatory cytokines, including IL-6 and IL-8 (Steffen *et al.*, 2000), even though their mRNA levels are increased after infection with wild-type *P. gingivalis* but not with its mutant deficient in gingipains (Baba *et al.*, 2002). In addition, there is accumulating evidence that gingipains are responsible for shedding and cleavage of CD14 receptors after treatment of human macrophage-like cells with the bacterium (Sugawara *et al.*, 2000; Duncan *et al.*, 2004). Taken together, we cannot rule out the possibility

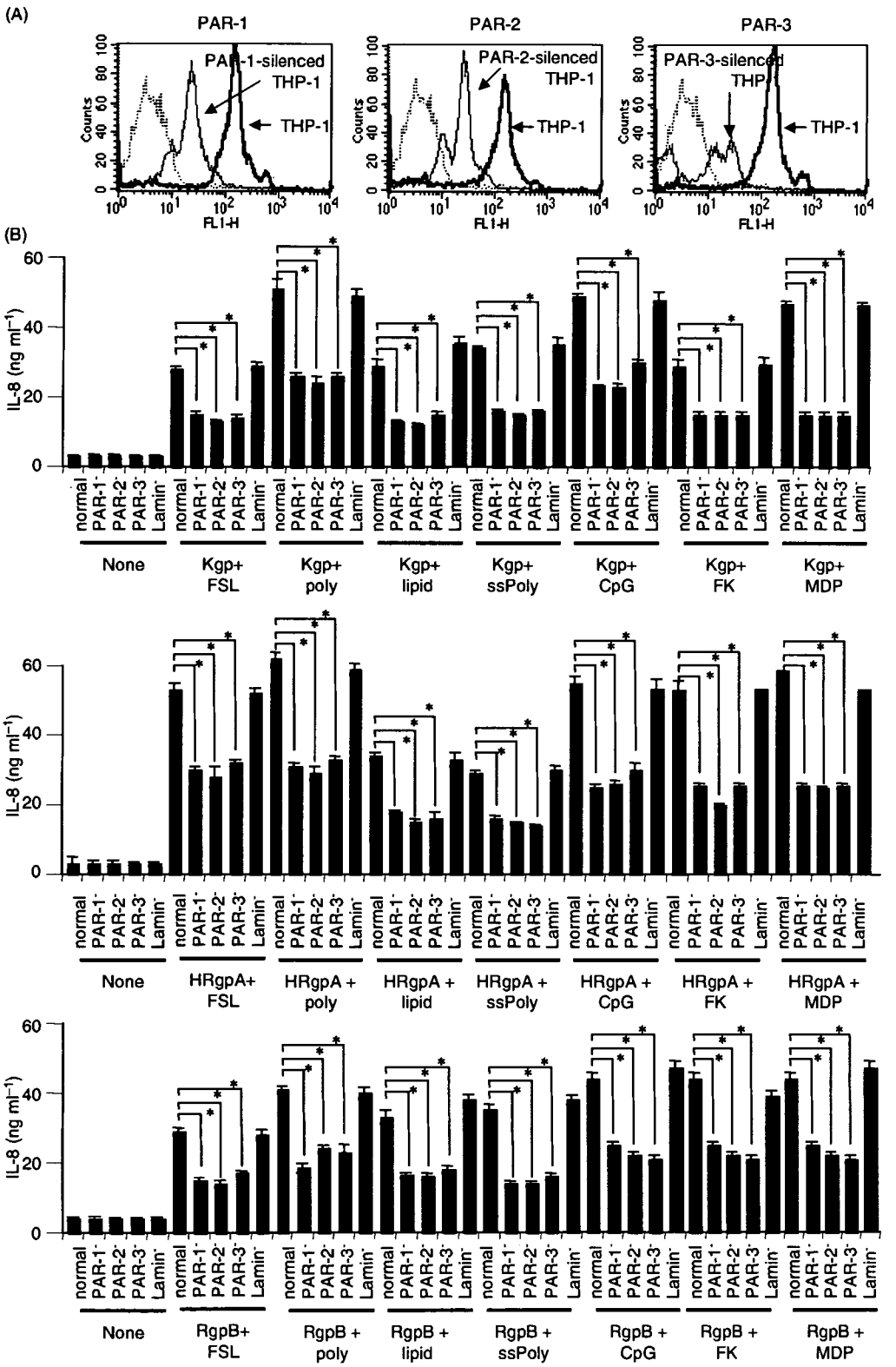

Fig. 6. Suppression of synergistic effects of gingipains and synthetic PAMPs in THP-1 cells treated with siRNA for PAR-1, PAR-2, or PAR-3.
A. THP-1 cells were transfected with siRNA targeting the PAR-1, PAR-2 or PAR-3 gene. Lamin A/C was used as a control. After 24 h, the cells were stained with Abs specific for PAR-1, PAR-2, or PAR-3 or, control IgG at 4 C for 30 min, followed by FITC-conjugated secondary Abs, and then subjected to flow cytometry.
B. THP-1 cells transfected with siRNA targeting the PAR-1, PAR-2, PAR-3 or Lamin A/C gene for 24 h were stimulated with 200 nM of a gingipain with either FSL-1 (1 nM), poly I:C (1 μg ml^{-1}), lipid A (10 ng ml^{-1}), ssPoly U (10 μg ml^{-1}), CpG DNA (1 μM), FK156 (100 μg ml^{-1}), or MDP (100 μg ml^{-1}) for 24 h. Concentrations of IL-8 in the culture supernatants were measured by ELISA, and values are the means ± SD for triplicate assays. $^{*}P < 0.01$ versus respective control (normal cells). Results representative of three different experiments are shown.

that gingipains may produce biologically inactive fragments of these cytokines, thereby contributing to an increased capacity of the bacterium to evade the host immune system mechanisms.

Porphyromonas gingivalis has been reported to possess TLR agonistic PAMPs: TLR4 agonist lipid A (Ogawa *et al.*, 2007), TLR2 agonist lipopeptides (Asai *et al.*, 2007), fimbriae (Asai *et al.*, 2001) and TLR9 agonist CpG DNA (Takeshita *et al.*, 1999). It should be noted, however, that TLR4-agonistic activity of *P. gingivalis* lipid A was exceptionally weak (Ogawa *et al.*, 2007). Furthermore, *P. gingivalis* PGN carries LL-DAP (Holt and Bramanti, 1991), which scarcely activated NOD1 unlike usual *meso*-DAP (Uehara *et al.*, 2006). Therefore, synergism between signalling via PARs induced by gingipains and PAMPs derived from the bacterium might be responsible for definite inflammatory responses induced by the bacterium in relation to pathogenesis of periodontitis. In addition, we may also speculate of the role of these processes in enhancement of host defence mechanisms.

Experimental procedures

Reagents

We used chemically synthesized PAMPs to avoid the influence of minor components in microbial preparations. The synthetic MDP, a NOD2 agonist, and *Escherichia coli* type lipid A (LA-15-PP), a synthetic TLR4 agonist, were purchased from the Protein Research Foundation Peptide Institute (Osaka, Japan). The *Mycoplasma salivarium* type diacyl lipopeptide FSL-1, a TLR2/6 agonist, was obtained from RMC microcollections (Tübingen, Germany). Poly I:C, a TLR3 agonist, and ssPoly U, a TLR7 agonist, were purchased from Sigma-Aldrich (St Louis, MO, USA). A conventional CpG DNA, CpG DNA 1826 [TCCAT GACGTTCCTGACGTT (CpG motif is underlined)], a TLR9 agonist, was purchased from SIGMA Genosys (Tokyo, Japan). FK156 (D-lactoyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly) (Kitaura *et al.*, 1982), a NOD1 agonist, was supplied by Astellas Pharmaceutical (Tokyo, Japan). Synthetic PAR-1AP (SFLLRN), PAR-2 AP (SLIGKV) and PAR-3AP (TFRGAP) were purchased from Takara (Otsu, Japan). All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Cells and cell culture

The human monocytic leukaemia cell line THP-1, supplied by the Health Science Research Resources Bank (Osaka, Japan), was cultured in RPMI 1640 medium (Nissui Seiyaku, Osaka, Japan) with 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified CO_2 atmosphere. The THP-1 cells were maintained in a logarithmic phase of growth (2×10^5 to 2×10^6) by passage every 3–4 days.

Human PBMCs were isolated from heparinized peripheral blood of healthy adult donors by Lympholyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada) gradient centrifugation at 800 *g* for 20 min at room temperature. The isolated PBMCs were washed three times with PBS and suspended in RPMI1640 medium.

Purification and activation of gingipains

HRgpA, RgpB and 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 (Von Jaggow), RgpB showed a single band with an apparent molecular weight of 48 kDa and the purity was > 95% as determined by laser densitometric scanning of the gel. HRgpA was composed of four major and one minor band on SDS-PAGE and each protein band was identified as a HRgpA component by N-terminal sequence analysis (Pike *et al.*, 1994). The amount of active enzyme in each purified gingipain was determined by active site titration using FPR-cmk and Z-FK-cmk for Rgps and Kgp respectively (Potempa *et al.*, 1997). The concentration of fully activated gingipains with cysteine was calculated from the amount of inhibitor needed for complete inactivation of the proteinases. Therefore, gingipain concentrations indicate active enzyme concentrations. The gingipains were activated in 0.2 M HEPES, 5 mM $CaCl_2$ and 10 mM cysteine, pH 8.0, at 37°C for 10 min, and then diluted with the medium or buffer. To block enzymatic activity, the activated gingipains were incubated with FPR-cmk or Z-FK-cmk for 10 min at room temperature before use.

Measurement of cytokines

The cells were collected and washed twice in PBS. They (2×10^5 cells per ml) were cultured in RPMI 1640 medium, supplemented with 10% FCS, in the presence or absence of a stimulant for 24 h in 96-well culture plates. Then, the culture supernatants were collected and the levels of IL-6, IL-8 and MCP-1 were measured with an enzyme-linked immunosorbent assay (ELISA) kit (OptEIA ELISA, BD Pharmingen, San Diego, CA, USA). The concentrations of a cytokine in the supernatants were calculated using the LS-Platemanager 2004 data analysis program (Wako Pure Chemical Industries, Osaka, Japan).

RNA interference

siRNAs (200 nM, the final concentration) for targeting the genes of PAR-1, PAR-2, PAR-3 or Lamin A/C were purchased from

Santa Cruz Biotechnology (Santa Cruz, CA, USA) and introduced into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The viability of the transfected cells was more than 95% when assessed with the Trypan blue exclusion test, and the cells did not change morphologically after the transfection.

Flow cytometry

Flow cytometric analyses were performed using a FACSCalibur flow cytometer and CELLQuest software (BD Biosciences, San Diego, CA, USA). Washed THP-1 cells were stained with mouse monoclonal antibodies specific for PAR-1, PAR-2 or PAR-3 (Santa Cruz), or isotype-matched control IgG at 4°C for 30 min, followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or goat IgG (Biosource International, Camarillo, CA, USA) at 4°C for a further 30 min. To calculate the percentage of positive cells, the baseline cursor was set at a channel that yielded less than 2% of the events as positive for the secondary Ab in the absence of the primary antibodies. Fluorescence to the right was counted as specific binding.

Data analysis

All experiments were performed at least three times to confirm the reproducibility of the results. Values are shown as the means ± SD from triplicate assays. The significance of differences was statistically evaluated by the one-way analysis of variance, using the Bonferroni or Dunnett method, and *P*-values less than 0.05 were considered significant.

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口腔細菌と歯周病
～口腔粘膜の自然免疫系～

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口腔細菌と歯周病
～口腔粘膜の自然免疫系～

上原　亜希子*1)　高田　春比古*2)

自然免疫系は昆虫からヒトに至る普遍的な機構であり，微生物に特有な構造(pathogen-associated molecular patterns：PAMPs) をパターン認識して生体防御を担っている。PAMPs を認識するレセプターとして，ヒトでは9種の Toll-like receptor (TLR) 系分子と細胞内で細菌細胞壁ペプチドグリカンを認識する NOD 系分子等が知られている。口腔粘膜の上皮細胞も TLR 系ならびに NOD 系分子を具備しているが，通常は炎症・免疫応答は抑制され，専ら抗菌因子産生にかかわっている。

Oral bacteria and periodontitis, with special reference to innate immune system in oral mucosa.

Tohoku University Graduate School of Dentistry, Department of Microbiology and Immunology

Akiko Uehara, Haruhiko Takada

Innate immune system is an ubiquitous system from insects to human and responsible for initial host defense against invasive pathogens, where pathogen-associated molecular patterns (PAMPs) are recognized by the pattern recognition molecules of hosts. Representative human receptors for PAMPs are nine Toll-like receptors (TLRs) and intracellular NOD molecules which recognize peptidoglycans of bacterial cell-walls. Oral epithelial cells express various TLRs and NODs so far examined, and these cells in normal condition produce antibacterial factors, but not proinflammatory factors, in response to various PAMPs to prevent bacterial invasion without excessive inflammatory responses.

はじめに

微生物に特有な構造 (pathogen-associated molecular patterns：**PAMPs**) をパターン認識して生体防御を営む自然免疫系の解明が進んでいる。生体膜上の Toll-like receptor (**TLR**) 系分子は菌体成分を，細胞内エンドソームの TLR 系分子は核酸の配列を認識して侵襲微生物のセンサーとなっている[1]。さらに，細胞内には NOD 系分子

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が存在し，細菌等の認識に携わっている[2]。

口腔には500～1,000種の細菌が生息すると言われている。これら細菌に恒常的に晒されている口腔上皮も，各種TLR系ならびにNOD系分子を発現している。しかし，通常の条件で口腔上皮細胞を各種PAMPsで刺激しても，炎症性サイトカインは誘導されない。本稿では，我々が研究を進めている口腔上皮組織の自然免疫系とその機能を中心に概説する。

口腔細菌叢

上述の通り，口腔に生息する細菌は極めて多様でありかつ量的にも莫大で，唾液1 mL当たりあるいはデンタルプラーク1 mg中に億オーダーの細菌が生息する。口腔の正常細菌叢はレンサ球菌(*Streptococcus*)等のグラム陽性菌が主体で，口腔のそれぞれの部位に特有のレンサ球菌が生息する。デンタルプラークにはミティス菌群(*S. mitis*, *S. sanguinis*等)，舌背にはサリバリウス菌群(*S. salivarius*等)，歯肉溝にはアンギノーザス菌群(*S. anginosus*等)が多い。なお，唾液の菌叢は主としてデンタルプラークと舌背に由来する。

歯周組織の炎症が進み，歯周ポケットが形成されると，菌量ならびに菌種は飛躍的に増加して，グラム陰性菌が主体となる。特に偏性嫌気性菌が増加して，歯周病(成人性歯周炎)の発症に深くかかわるとされている。歯周病関連菌の*Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*等はいずれも*Bacteroides*類縁の菌である。その他，鞭毛を有して運動性の*Campyrobacter rectus*も著名である。一方，まれに発症する若年性歯周炎には*Actinobacillus actinomycetemcomitans*(グラム陰性の通性嫌気性菌)がかかわるとされている[3]。

TLR系によるPAMPsの認識

細菌表層にはPAMPsが局在しており，それぞれのPAMPsを認識するTLR系分子が解明されている。すなわち，マイコプラズマを除くほぼすべての細菌に存在するペプチドグリカン(PGN)，細菌に普遍的なリポプロテイン，多くのグラム陽性菌に存在するリポタイコ酸(LTA)等は主としてTLR2によって，グラム陰性菌外膜成分の内毒素性リポ多糖(LPS)ないしリピドAはTLR4によって，細菌の運動器官である鞭毛はTLR5によって認識される。さらに細菌やウイルスに特徴的なデオキシリボ核酸(DNA)やリボ核酸(RNA)配列は細胞内エンドソームに分布するTLR分子によって認識される。すなわち，ウイルスの二本鎖RNAはTLR3により，一本鎖RNAはTLR7/8により，細菌性CpG DNAはTLR9によって認識される(**図1**)[1]。

歯周病原菌と目される*P. gingivalis*を始めとする*Bacteroides*類縁菌のリピドAは共通した構造を有し，いずれも生物活性が極めて弱い。これは特異なリピドA構造のためにTLR4に認識されにくいためと考えられる。すなわち，これらの菌はグラム陰性菌センサーとしてのTLR4を回避して歯周組織に生息し続けるものと考えられる。

*P. gingivalis*のLPS(リピドA)がTLR2活性を発揮するとの多数の報告がある。しかし，小川知彦グループ(朝日大学)の研究は，これらTLR2活性は同菌LPS(リピドA)から通常の方法では分離できないリポペプチドに起因することを強く示唆している[4]。ちなみに同グループは，*P. gingivalis*繊毛もTLR2に作用すると報告している[5]。歯周病ならびにそれにしばしば随伴する慢性の心血管疾患の発症に，TLR2活性が深くかかわるとの知見と併せ考えると興味深い。

NOD系分子によるPAMPsの認識

PGNが示す多彩な生物活性の多くがムラミルジペプチド(MDP)によって担われていることから，MDPはアジュバント活性を始めとするPGN

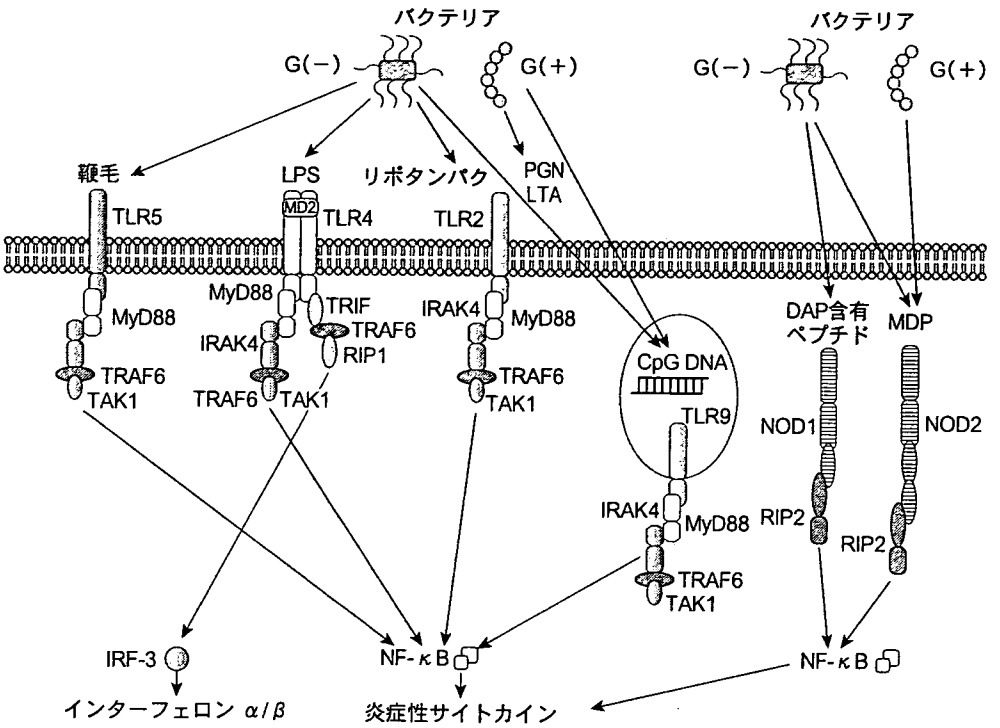

図1　TLR系およびNOD系を介する細菌性PAMPsの認識機構

宿主細胞は侵襲細菌に共通な菌体成分や特有の核酸配列PAMPsをTLR系ならびにNOD系分子を介して認識し，自然免疫応答を示す。

DAP：ジアミノピメリン酸，DNA：デオキシリボ核酸，IRAK：IL-1R-associated kinase，IRF-3：interferon regulatory factor-3，LPS：リポ多糖，LTA：リポタイコ酸，MDP：ムラミルジペプチド，MyD88:myeloid differentiation factor 88，NF-κB:nuclear factor-κB，PAMPs:pathogen-associated molecular patterns，PGN:ペプチドグリカン，RIP:receptor interacting protein，TAK:TGFβ-activated kinase，TLR:Toll-like receptor，TRAF:TNF receptor-associated factor，TRIF:Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β

（文献1より作成）

の最小有効構造と目されてきた（**図2**）[6,7]。またPGNの別の活性構造として*meso*-diaminopimelic acid（*meso*-**DAP**）を含むペプチドフラグメントも化学合成され，MDPと同様の生物活性が報告されている（**図2**）[8,9]。2003年になって，米・仏2つの研究グループによりMDPがNOD2を介して作用するとの報告がなされ[10,11]，ついでNOD1が*meso*-DAP含有ペプチドを認識することが証明された（**図2**）[12,13]。

最近我々は，*meso*-DAP単独でも口腔を始めとするさまざまなヒト上皮細胞をNOD1を介して活性化することを証明した[14]。合成化合物を供試した研究の結果，*meso*-ランチオニンも*meso*-DAPと同等の作用を示す。さらに*meso*-DAPの光学異性体のLL-DAPは弱い活性を示すが，DD-DAPは全く活性を欠いていた。ちなみに，口腔に生息するグラム陰性菌の多くは*meso*-DAP型PGNを有するが，*P. gingivalis*のPGNはLL-DAP[15]を，*Fusobacterium nucleatum*のPGNは*meso*-ランチオニン[16]を有している。なお，DD-DAPを有する細菌の報告は無い。

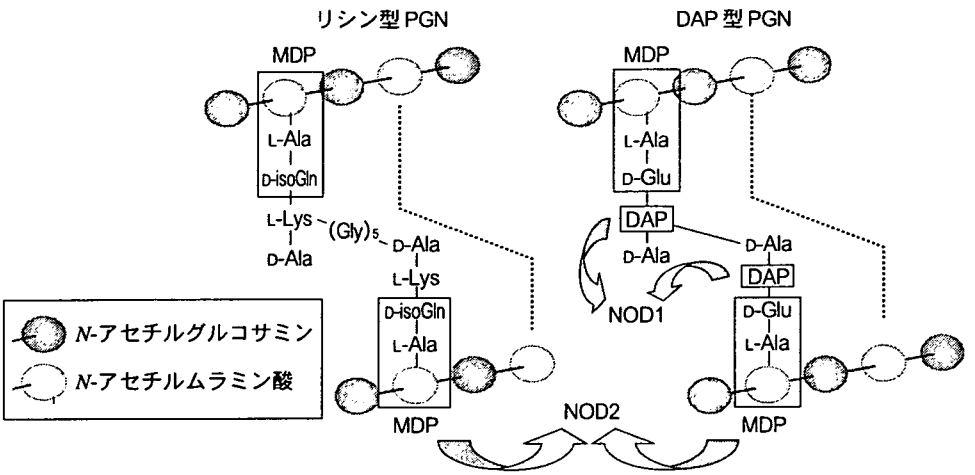

図2　PGNの構造とその活性部位

大部分のグラム陽性菌のPGNはリシン型，多くのグラム陰性菌と結核菌など一部のグラム陽性菌のPGNはDAP型である。PGNのMDP部分とDAP含有ペプチド部分を認識する細胞内レセプターがNOD2とNOD1である。

DAP：ジアミノピメリン酸，MDP：ムラミルジペプチド，PGN：ペプチドグリカン

（筆者ら作成）

口腔上皮の自然免疫系レセプター発現

ヒト正常歯周組織のTLR系とNOD系分子を免疫染色法で検討したところ，上皮組織にNOD1ならびにNOD2の強い発現と，TLR2ならびにTLR4の明確な発現が認められた。さらに炎症歯肉上皮では，TLR2とTLR4が細胞表層に強く局在することが明らかになった[17][18]。培養口腔上皮細胞を供試して詳細に検討したところ，すべてのTLR分子（TLR1～9）を遺伝子ならびにタンパクレベルで発現していた（Uehara et al，未発表）。

NOD系およびTLR系刺激によるヒト口腔上皮細胞からの抗菌因子産生

ヒト口腔上皮系細胞に発現しているTLR系ならびにNOD系分子を対応するリガンドで刺激しても，炎症性サイトカインを産生しない（**図3**）[17][18]。細菌に常に曝されている口腔上皮細胞が炎症性サイトカインを産生しないのは，過剰な炎症応答を避けるシステムとも考えられる。一方，同じ刺激に応じてβ-defensin 2やPGN認識タンパク（**PGRP**）といった抗菌物質の産生は強力に誘導される（**図3**）[17][18]。これらの知見は口腔上皮細胞上に発現している各種TLR系ならびにNOD系は自然免疫系分子のレセプター機能を具備していることを示している。

おわりに

これまで，口腔上皮細胞の菌体成分に対する不応答性については，特定のTLR系分子を欠如しているとの解釈が一般的であった。我々の研究によって，ヒト口腔上皮細胞は，各種TLR系およびNOD系分子を発現しているにもかかわらず，対応するリガンドで刺激しても全く炎症性サイトカイン応答を示さないことが明らかになった。しかし，発現しているTLR系分子が機能を欠如している訳ではなく，対応するリガンド刺激に応じて口腔上皮細胞は高レベルの抗菌因子を産生する。

一方，各種炎症性サイトカイン等でプライムされた口腔上皮細胞は，菌体成分刺激に応じて高レ