BACTERIAL PATHOGENICITY

Activation of human gingival epithelial cells by cell-surface components of black-pigmented bacteria: augmentation of production of interleukin-8, granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor and expression of intercellular adhesion molecule 1

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Black-pigmented anaerobic bacteria, such as Porphyromonas gingivalis and Prevotella intermedia, are amongst the predominant bacteria in periodontal pockets and have been implicated in periodontal diseases. To elucidate the roles of gingival keratinocytes, which are the first cells encountered by oral bacteria in periodontal diseases, human gingival keratinocytes in primary culture were stimulated with cell-surface components of P. gingivalis and P. intermedia. A glycoprotein fraction from P. intermedia (PGP) clearly augmented the release of interleukin-8, granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor, as determined by enzyme-linked immunosorbent assay. This PGP also induced expression of intercellular adhesion molecule-1 (ICAM-1), as determined by flow cytometry. The augmentation of mRNA expression for these molecules was also confirmed by reverse transcription PCR. In contrast, lipopolysaccharide (LPS) from P. intermedia and Escherichia coli was completely inactive in these assays. LPS fraction and purified fimbriae from P. gingivalis exhibited weak activities. Cytokine production and ICAM-1 expression by gingival keratinocytes might cause accumulation and activation of neutrophils in the epithelium and, therefore, may be involved in the initiation and development of inflammation in periodontal tissues.

Introduction

Adult periodontitis is a highly destructive chronic inflammatory disease. Oral black-pigmented anaerobic bacteria (BPB), such as Porphyromonas gingivalis and Prevotella intermedia, are amongst the predominant bacteria in periodontal pockets of patients with periodontitis and have been implicated in periodontal diseases [1, 2]. Possible virulence factors of BPB in relation to the pathogenesis of periodontal diseases have been reviewed [3, 4] and the immunobiological activities of cell-surface components, such as lipopolysaccharide (LPS) and fimbriae have been investigated. LPS from BPB has been reported to exhibit unique bioactivities that differ from those of common LPS prepared from Enterobacteriaceae, such as Escherichia coli and Salmonella spp. [5, 6]. Immunocompetent cells from animals and human peripheral blood cells were used in the initial studies, and subsequently human fibroblasts derived from periodontal tissues were examined. In a previous study, a novel bioactive glycoprotein fraction, designated as Prevotella glycoprotein (PGP), was isolated from P.
intermedia ATCC 25611 by the hot phenol-water extraction method [7]. PGP has been shown to stimulate lymphoid cells from genetically LPS-resistant mice and human gingival fibroblasts to secrete inflammatory cytokines, whereas purified LPS prepared from the bacterium with phenol-chloroform-petroleum ether (PCP) mixture was devoid of such activities. Therefore, it has been proposed that the unique bioactivities of BPB LPS reported to date may be attributable to PGP or PGP-like material(s) co-existing with the BPB LPS extracted by the phenol-water extraction method.

The first cells encountered by bacteria are epithelial cells, and the predominant cell type in the gingival epithelium is the keratinocyte [8]. Skin keratinocytes and keratinocytic cell lines produce various cytokines and growth factors [9] and some studies [10, 11] have also shown cytokine production by human oral keratinocytes and related cell lines in response to stimulation with oral bacteria. However, the cytokine repertoire of gingival keratinocytes in response to specific oral bacterial component(s) is not clear at present.

The aim of the present study was to elucidate the possible involvement of gingival keratinocytes in the host response to bacterial stimuli. The cytokine response and expression of an adhesion molecule by human gingival keratinocytes in primary culture after stimulation with various cell-surface components of BPB was examined in relation to the initiation and establishment of periodontal disease.

Materials and methods

Bacterial components and cytokines

PGP was prepared from Pr. intermedia ATCC 25611 as described previously [7]. Briefly, lyophilised bacterial cells were extracted twice with phenol-water at 67°C for 20 min. The pooled extract in the water phase was dialysed against distilled water and ultracentrifuged at 140,000 g for 3 h to remove LPS as a sediment. The supernate was lyophilised and a portion was dissolved in sodium deoxycholate 0.2%-100 mM Tris-5 mM sodium EDTA (pH 8.5) which was then subjected to chromatography on Sephacry G 100 to prepare the final fraction, PGP. LPS was extracted from same bacteria with a PCP mixture according to the method of Galanos et al. [12] before further treatment with NP1 nuclease (Yamasa, Choshi, Japan) and re-chromatographed on Sephacry G 100 to prepare the final fraction, PGP. LPS was extracted from same bacteria with a PCP mixture according to the method of Galanos et al. [12] before further treatment with NP1 nuclease and pronase E from Streptomyces griseus attached to carboxymethyl cellulose beads (Sigma). The purified LPS was mitogenic on spleenocytes from C3H/HeN but not those from C3H/HeJ mice [7]. Fimbriae were isolated from P. gingivalis 381 and purified as described previously [13]. LPS could not be extracted from P. gingivalis 381 by the PCP method. Therefore, an LPS fraction was prepared from P. gingivalis 381 by the conventional hot phenol-water extraction method [7]. Ultrapurified LPS from Salmomella choleraeus subsp. choleraeus Abortus-equ [14] was a gift from C. Galanos (Max Plank Institut für Immunbiologie, Freiburg, Germany). Human natural interferon (IFN)-γ (antiviral activity, 8.0 × 10^8 IU/mg of protein) was provided by Hayashibara Bioscience Institute (Okayama, Japan) and rHuIL-1α (lymphocyte activating activity, 2.3 × 10^5 U/mg of protein) was supplied by Dainippon Pharmaceutal (Osaka, Japan).

Cells and cell culture

Human gingival epithelial cells were prepared from the explants of normal human gingival tissues of 7–10 year-old donors with their own and their parents’ informed consent. The explants were cut into pieces and cultured in tissue culture dishes 100 mm in diameter (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA) in keratinocyte serum-free medium (Gibco BRL, Grand Island, NY, USA) containing bovine pituitary extract 0.05% v/v and human epidermal growth factor 1-51 (recombinant from E. coli, 820 μm) supplemented with kanamycin (Meiji Seiyaku, Tokyo, Japan) 200 μg/ml with a medium change every 4 days for 15–20 days until subconfluent cell monolayers were formed. Human gingival keratinocytes obtained in this manner were characterised as keratinocytes on the basis of immunostaining with anti-cytokeratin type I and II antibodies (AEI+AE3 mouse IgG1 MAb; Progen Biotechnik GmbH, Germany) [15]. The cells were also confirmed to be devoid of CD14, a receptor for LPS and various bacterial cell-surface components by flow cytometry and reverse transcription PCR (RT-PCR) (data not shown). The cells were used within three passages.

Flow cytometry

Flow cytometric analyses were performed with a fluorescence-activated cell sorter (FACS) (FACScan; Becton Dickinson, Mountain View, CA, USA). For immunofluorescent staining, confluent human gingival keratinocytes in six-well plates (Falcon) at a density of 5 × 10^5 cells/3 ml/well were incubated with or without various test materials for 48 h, then cells were collected by trypsinisation and washed in PBS. The cells were stained with anti-CD54 (intercellular adhesion molecule-1, ICAM-1) monoclonal antibody (MAb) directly conjugated with phycoerythin (PE) (mouse IgG2b, obtained from Becton Dickinson) at 4°C for 30 min. Murine IgG2b (Coulter, Miami, FL, USA) was used as a control isotype-matched antibody to exclude the possibility of non-specific binding of the anti-CD54 MAb. To calculate the percentage of positive cells, the baseline cursor was set at a channel that yielded <2%
of the events positive with a second antibody control. Fluorescence to the right was counted as specific binding.

**Enzyme-linked immunosorbent assay (ELISA)**

For the detection of cytokines in the human gingival keratinocyte culture supernate, the confluent cells (2.4 × 10²/200 µl/well) in 96-well plates (Falcon) were incubated with or without various test materials for 24–72 h. Cultures were performed in triplicate and the concentrations of cytokines in the supernates were determined with ELISA kits for interleukin (IL)-1α, IL-1β, IL-2, IL-6, IL-8, IL-15, tumour necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Biosource, Camarillo, CA, USA) and granulocyte colony-stimulating factor (G-CSF) (Japan Immunoresearch Lab. Co. Ltd, Takasaki, Japan).

**RT-PCR**

Total cellular RNA was extracted from cells with Iso- gen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. Reverse transcription of RNA samples to cDNA was performed with Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and oligo(dT)₁₂–₁₈ primer (Gibco BRL). To transcribe total RNA into cDNA, 2.5 µg of RNA, 0.25 µg oligo(dT)₁₂–₁₈ primer, 500 U of Moloney murine leukemia virus reverse transcriptase, 10 µl of 5× first strand buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs) and 10 mM dithiothreitol (DTT) were added to a total volume of 50 µl. The reaction mixture was incubated for 1 h at 37°C followed by 5 min at 95°C. The primers used for PCR had the following sequences: IL-8, forward 5’-GATTCGGAGTGGACCA CACT-3’ and reverse 5’-TTCCTCGTGCAATATCT AGG-3’; ICAM-1, forward 5’-ACCATGGAGCCAT TTTCG-3’ and reverse 5’-GAGAAGGAGTGTGG CCATA-3’; G-CSF, forward 5’-CTGGCTTGAGCCA ACTCCATA-3’ and reverse 5’-GGATTCACAACC ATGTCCCAA-3’; GM-CSF, forward 5’-GTAGAGAC ACTGCTGGTGAAG-3’ and reverse 5’-TCTGTATCA GGTCAGTTGG-3’; and human glyceraldehyde-3- phosphate dehydrogenase (GAPDH) forward 5’- TGAAGGTCGGATGCAAAGCTTGGG-3’ and reverse 5’-CATGTTGGCCATGAGTTCCACCAAC-3’. The primers for IL-8, ICAM-1, G-CSF, GM-CSF and GAPDH were constructed to generate fragments of 422, 276, 871, 456 and 983 bp, respectively. The PCR mixture contained 5 µl of the cDNA mixture, 2 µl of 10× PCR buffer, 0.2 mM dNTPs, 50 pmol of each primer and 0.1 µl of Ex taq DNA polymerase (Takara, Tokyo, Japan) in a total volume of 20 µl. Amplification was performed in a model MP TP3000 PCR thermal cycler (Takara) as follows: 25 cycles of denaturation at 94°C for 1 min, annealing at 63°C for IL-8, at 65°C for ICAM-1, at 65.7°C for G-CSF, at 66.7°C for GM-CSF and at 60°C for GAPDH, for 1 min; extension at 72°C for 1 min and final extension at 72°C for 3 min.

Amplified samples were visualised on agarose 2.0% gels stained with ethidium bromide and photographed under UV illumination. The bands on the photographs were scanned and then analysed with Image Master ID (Pharmacia Biotech, Uppsala, Sweden). Results were expressed as relative mRNA accumulation corrected with GAPDH mRNA as an internal standard.

**Miscellaneous**

Most experiments were performed more than three times and representative results are presented. In cytokine assays, based on the mean value and SD, the statistical significance of differences between each test and the respective control was examined by one-way analysis of variance (ANOVA) by the Bonferroni or Dunn method; p values <0.05 were considered significant.

**Results**

**Cytokine induction by BPB cellular components in human gingival keratinocytes**

The time-course of IL-8 secretion by human gingival keratinocytes in response to the PGP fraction from *P. intermedius* was examined (Fig. 1). The PGP fraction (10 µg/ml) induced IL-8 secretion from 24 to 72 h at a similar level; therefore, cultivation for cytokine production was done for 24 h in the following experiments. The PGP fraction enhanced secretion of IL-8.

![Fig. 1. IL-8 secretion by human gingival keratinocytes stimulated with the PGP fraction from *P. intermedius*. Primary cultures of human gingival keratinocytes were incubated with PGP (10 µg/ml; ■) or medium alone (○) for 24–72 h (*p <0.01).](image-url)
G-CSF and GM-CSF in a dose-dependent manner and the maximum levels induced by PGP 10 μg/ml were similar to or higher than respective levels of cytokines induced by rHuIL-1α and HufN-γ (Fig. 2). Three LPS specimens were almost inactive in this respect, except that *P. gingivalis* LPS significantly induced G-CSF production when the highest concentration (10 μg/ml) was used. The purified filaments of *P. gingivalis* at 100 μg/ml significantly induced IL-8 and G-CSF. The supernates of the keratinocyte cultures did not contain detectable levels of IL-2, IL-6, IL-15 and TNF-α. IL-1α and IL-1β were detected but were not upregulated by the bacterial components tested (data not shown).

**Effect of BPB cell-surface components on ICAM-1 expression by human gingival keratinocytes**

HufN-γ induced ICAM-1 expression on almost 100% of gingival keratinocytes (Fig. 3). Among the bacterial cell-surface components, *Pr. intermedia* PGP (10 μg/ml) and *P. gingivalis* LPS (10 μg/ml) augmented ICAM-1 expression, from c. 5% in medium alone to c. 50 and 30%, respectively. *P. gingivalis* filaments (100 μg/ml) exhibited weak activity (12.5%) whereas purified LPS (10 μg/ml) from *Pr. intermedia* lacked activity like Salmonella LPS (data not shown).

**mRNA expression for IL-8, G-CSF GM-CSF and ICAM-1 by gingival keratinocytes stimulated with BPB cell-surface components**

To assess the specific induction of these molecules at the mRNA level, RT-PCR was performed with the RNA isolated from gingival keratinocytes. First, the time-course of IL-8 mRNA expression induced by the PGP fraction (10 μg/ml) was assessed. The peak expression was observed after stimulation for 8 h (Fig. 4); therefore, RT-PCR was performed after cultivation for 8 h in the following experiments. Subsequently, the expression of various cytokines and adhesion molecules was examined. The levels of IL-8, G-CSF, GM-CSF and ICAM-1 mRNAs from cells treated with *Pr. intermedia* PGP, *P. gingivalis* filaments and *P. gingivalis* LPS were increased as compared with those from untreated control cells (Figs. 5 and 6). In contrast, the cytokine and ICAM-1 mRNAs were scarcely detected in cells stimulated with *Pr. intermedia* LPS or were detected at intensities similar to those in medium alone (control) by RT-PCR (Fig. 5).

**Discussion**

BPB, in particular *P. gingivalis* and *Pr. intermedia*, possess various virulence factors on their cell surfaces, such as LPS [5, 6] and filaments [16, 17]. The immunobiological activities of these factors have been studied extensively in relation to the pathogenesis of periodontal diseases. The present study has demonstrated that *Pr. intermedia* PGP fraction stimulated gingival keratinocytes to increase production of IL-8, G-CSF and GM-CSF and augmented expression of ICAM-1. In contrast, LPS from *Pr. intermedia* as well as Salmonella completely lacked these activities. These results are not surprising because gingival keratinocytes do not have CD14, the main receptor for LPS [18] on cell-surface. In this study, exogenous soluble CD14 was not added because serum-free medium was used throughout the experiments. However, it must be noted that human gingival keratinocytes in primary culture

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<td>Medium alone</td>
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<td>IFN-γ (200 U/ml)</td>
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**Fig. 2. Cytokine secretion by human gingival keratinocytes stimulated with cell-surface components from *Pr. intermedia* and *P. gingivalis*.** Primary cultures of human gingival keratinocytes were incubated with stimulant or medium alone for 24 h (*p <0.05, **p <0.01).
Fig. 3. ICAM-1 expression on human gingival keratinocytes stimulated with various cell-surface components of BPB. Human gingival keratinocytes were incubated with stimulant or medium alone for 48 h: a, medium alone (4.9%); b, IFN-γ (98.7%); c, Salmonella LPS (5.1%); d, P. intermedia PGP (49.8%); e, P. gingivalis fimbriae (12.5%); f, P. gingivalis LPS (30.5%).

Fig. 4. The time-course of mRNA expression by human gingival keratinocytes after stimulation with PGP fractions from P. intermedia. Human gingival keratinocytes were cultured with or without test material (10 μg/ml) for 4–12 h. PCR products were electrophoresed on agarose 2% gels and visualised by staining with ethidium bromide (a). Blots were further quantified with an imaging analyser and results are expressed as relative mRNA accumulation corrected relative to GAPDH mRNA as an internal standard (b).

and oral epithelial cell lines do not respond to LPS even in the presence of soluble CD14 [19]. It has also been reported that the primary culture and cell line culture in experiments [19] involving low and high (physiological) concentrations of calcium, respectively, indicate that calcium concentration does not influence the responses of epithelial cells to LPS. Therefore, the observed activities of P. gingivalis LPS might be attributable to PGP or PGP-like material possibly present in the LPS fraction. In this context, previous research has shown that a synthetic compound mimicking P. gingivalis lipid A, unlike the natural counterpart, did not activate macrophages from C3H/HeJ mice [20].

P. gingivalis and P. intermedia are capable of invading human gingival epithelial cells and oral epithelial cell lines [21, 22]. P. gingivalis fimbriae may be involved in invasion of the bacteria into the epithelium, because a fimbriae-deficient mutant of P. gingivalis was less invasive than the parent strain, and the presence of fimbriae receptor on gingival epithelial cells has been suggested [23, 24]. Gingival epithelial cells invaded by P. gingivalis show reduced production of IL-8 in response to other bacterial stimuli [25]. Although binding of P. gingivalis fimbriae on gingival keratinocytes has been suggested by several investigators [23, 26], the present report appears to be the first study to show that P. gingivalis fimbriae activated human gingival keratinocytes to enhance production of IL-8. This finding is not in conflict with the above inhibitory effect of fimbriate P. gingivalis cells on cytokine production by the gingival keratinocytes [25], because the latter report only indicated fimbriae-dependent invasion of the bacterium and did not show that fimbriae themselves inhibited the function of gingival epithelial cells.
Fig. 5. Induction of mRNA expression by human gingival keratinocytes after stimulation with PGP and LPS fractions from P. intermedia. Human gingival keratinocytes were cultured with or without test materials for 8 h. PCR products were electrophoresed on agarose 2% gels and visualised by staining with ethidium bromide (A). Blots were further quantified with an imaging analyser and results are expressed as relative mRNA accumulation corrected relative to GAPDH mRNA as an internal standard (B). M, medium alone; P, PGP; L, LPS; a, IL-8; b, G-CSF; e, GM-CSF; d, ICAM-1; e, GAPDH.

Fig. 6. Induction of mRNA expression by human gingival keratinocytes after stimulation with fimbriae and LPS fractions from P. gingivalis. Human gingival keratinocytes were cultured with or without test materials for 8 h. PCR products were electrophoresed on agarose 2% gels and visualised by staining with ethidium bromide (A). Blots were further quantified with an imaging analyser and results are expressed as relative mRNA accumulation corrected relative to GAPDH mRNA as an internal standard (B). M, medium alone; F, fimbriae; L, LPS; a, IL-8; b, G-CSF; e, GM-CSF; d, ICAM-1; e, GAPDH.

As mentioned above, many previous studies demonstrated that skin keratinocytes produced various cytokines and growth factors and expressed adhesion molecules on their surface as a result of stimulation with environmental stimuli such as UV light, and were thought to actively participate in inflammatory and immunological responses [27]. However, until recently, gingival keratinocytes have been thought to act as a mechanical barrier against exogenous stimuli. The production of inflammatory cytokine by gingival keratinocytes in response to bacterial stimuli was examined here. Unexpectedly, most cytokines were not detected or were not upregulated by bacterial stimuli. The results presented here demonstrate in a primary cell culture system that human gingival keratinocytes secrete IL-8, G-CSF and GM-CSF and express ICAM-1 after stimulation with cell-surface components from BPB. In the inflamed gingiva, IL-8 has been detected mainly in the epithelial layer [28]. IL-8 secreted by gingival epithelial cells might act as a chemo-attractant for neutrophils and T cells [29, 30]. These accumulated cells might express LFA-1 on their cell surface and attach to epithelial cells via ICAM-1 and LFA-1 interaction, then infiltrate into the gingival epithelium [8, 27]. In fact, ICAM-1 and IL-8 mRNA expression are topographically associated with the area of neutrophil migration in the junctional epithelium [31]. IL-8-activated neutrophils show a respiratory burst to produce superoxide [30]. G-CSF and GM-CSF are also capable of activating neutrophils to augment superoxide anion generation in response to the bacterial chemo-attractant N-formylmethionyl-leucyl-phenylalanine (FMLP) [32, 33]. These responses of neutrophils might in turn injure gingival epithelial cells [34] and might result in the initiation and establishment of periodontal diseases.
GINGIVAL KERATINOCYTE ACTIVATION BY BF2


