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Expression of β-defensin-2 in human gingival epithelial cells in response to challenge with *Porphyromonas gingivalis in vitro*

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Background and Objective: Human β-defensin-2 (hBD-2) is an antimicrobial peptide that is produced by epithelial cells after stimulation with microorganisms and inflammatory mediators. Compared with gram-positive bacteria, gram-negative bacteria, which are typically detected in the periodontal pockets in periodontitis, elicit a stronger antibacterial peptide response of hBD-2 by epithelial cells. The purpose of this study was to investigate the expression of hBD-2 and relationships between it and inflammatory mediators in human gingival epithelial cells (HGEC) in response to challenge with *Porphyromonas gingivalis in vitro*.

Material and Methods: mRNA expression of hBD-2 in HGEC stimulated with or without *P. gingivalis* was assessed using a semiquantitative reverse transcription–polymerase chain reaction. Primary cultured HGEC were activated by live *P. gingivalis*, and inflammatory cytokine production was examined using an enzyme-linked immunosorbent assay.

Results: The level of hBD-2 mRNA in HGEC treated with *P. gingivalis* increased with exposure time. After 48 h, the mRNA in *P. gingivalis* was significantly increased compared with that in control HGEC. The interleukin-8 production rate was much greater in stimulated HGEC than in the control HGEC, almost always showing a significant difference after 3 h. The production of interleukin-1β was not increased as much as that of interleukin-8.

Conclusion: These findings suggest that the expression of hBD-2 in HGEC is *P. gingivalis*-dependently induced and is likely to be connected with the initial stage of the inflammatory response.

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Periodontal disease is among the infectious diseases caused by microorganisms that colonize the tooth surface at or below the gingival margin (1). These microorganisms, especially gram-negative anaerobic periodontal bacteria, and their secretions, lead to the destruction of periodontal tissues such as connective tissue and alveolar bone surrounding the teeth. *Porphyromonas gingivalis* is a representative periodontal bacterium that is detected in specimens from almost all adult periodontitis patients (2). Oral sulcular epithelial cells, which are first and directly invaded by these periodontal bacteria, act not only as mechanical barriers but also as immunological

barriers by producing various cytokines against the periodontal bacteria. In addition, these cells manifest the innate immune mechanisms of epithelial cells, found elsewhere in our bodies, providing the initial host defensive responses, for example epithelial cell production and release of cytokines and other molecules that possess antimicrobial effects and induce leucocyte production.

One of the other recognized host-defense systems involving the innate immune response of gingival epithelial cells exposed to the external environment is the production of human β-defensin (hBD) as a natural antimicrobial peptide. hBD, a major antimicrobial peptide derived from the epithelial cell, detects host cells by forming pores in the cytoplasmic membrane of microorganisms (3). One type of defensin – human β-defensin-2 (hBD-2) – is a cysteine-rich cationic low-molecular-weight antimicrobial peptide that was discovered in psoriatic lesional skin and is suggested to be involved in cutaneous defense and inflammation. hBD-2 exhibits a stronger potential antimicrobial activity against gram-negative organisms, such as *Escherichia coli* and *Pseudomonas aeruginosa* and the yeast, *Candida albicans*, than against gram-positive organisms such as *Staphylococcus aureus* (3–5). Generally speaking, hBD-2 is poorly expressed in normal epithelial cells, but is expressed by epithelial cells stimulated with microorganisms such as *P. aeruginosa*, *Helicobacter pylori and Fusobacterium nucleatum*, or by cytokines such as tumor necrosis factor-α and interleukin-1β (3–5). Thus, hBD-2 plays a role in the initial infection defense system at the epithelial cell level, such as skin and gingiva.

Differences in expression of the *hBD* gene between healthy subjects and those with periodontal disease were recently reported (6), and expression of the *hBD-2* gene was described in human gingival epithelial cells (HGEC) stimulated with *Actinobacillus actinomycetemcomitans* (7). However, expression of the *hBD-2* gene in periodontal pocket epithelial cells stimulated with live *P. gingivalis in situ* in the oral cavity has not been investigated. Whereas a significant relationship between antimicrobial peptides derived from epithelial cells and neutrophils has been described (8), there are no reports for *P. gingivalis*. In this study, we investigated *hBD-2* gene expression by periodontal pocket junctional epithelial cells stimulated with live *P. gingivalis* and compared their expression with the expression of inflammatory cytokines around neutrophils secreted from these cells at various time points during stimulation.

Material and methods

Primary culture of HGEC

The present study was approved by the Ethics Committee of Osaka Dental University (Approval No. 040519). Human gingival tissue specimens were obtained from 10 patients, with their informed consent, during periodontal surgical therapy. All of these tissue specimens were from patients with severe periodontitis accompanied with a periodontal pocket of > 5 mm and pocket epithelium. We cultured HGEC from tissue specimens according to the methods reported by Matsuyama *et al.* (9). Briefly, gingival tissues were cut into 1-mm^3 pieces and washed in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Life Technologies, Grand Island, NY, USA) containing 500 U/ml penicillin, 500 μg/ml streptomycin, and 1.25 μg/ml fungizone (Camblex Bio Science Walkersville Inc., Walkersville, MD, USA). The small gingival pieces were placed in a six-well tissue-culture plate coated with type I collagen (Asahi Techno-glass Inc., Tokyo, Japan) and then dehydrated in the incubator for 10 or 15 min. The gingival pieces were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and 1% antiobiotics until outgrowth of epithelial cell layers was observed. The culture medium was then exchanged for keratinocyte-SFM (Gibco-BRL), supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor and 1% antibiotics. The medium were changed every 3 d, and the cells were harvested and seeded until they reached subconfluence. In this study, three cultures were used.

Preparations of periodontal bacterium

In the present study, a *P. gingivalis* strain from the American Type Culture Collection (ATCC33277; Manassas, VA, USA), distributed courtesy of Sunstar Inc. (Takatsuki, Osaka, Japan), was cultured in brain–heart infusion medium (BHI; Difco, Sparks, MD, USA) containing 0.5% yeast extract (BBL, Sparks, MD, USA), 0.001% vitamin K_1 (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) and 0.0005% hemin (Sigma, St Louis, MO, USA) after culture in Center for Disease Control (CDC) blood agar medium. The bacterial suspension was mixed with Hank's balanced salt solution containing 0.1% gelatin (Gel-HBSS) (Gibco-BRL), and then adjusted to an optical density (OD) of 0.3 at 540 nm using a spectroscopic photometer (Hitachi Inc., Saitama, Japan).

Cell culture with periodontal bacteria

HGEC were harvested and seeded at a density of 5×10^4 cells/well into type I collagen-coated 24-well tissue culture plates. After 2 d, a mixed bacterial suspension was diluted tenfold in the cell culture medium. HGEC, with or without *P. gingivalis*, were cultured for 1, 3, 6, 24, 48, or 72 h and examined by reverse transcription–polymerase chain reaction (RT–PCR). Their culture supernatants were investigated by enzyme-linked immunosorbent assay (ELISA).

Detection of mRNA expression of hBD-2

Total RNA from each culture was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and quantified by spectrometry (Shimadzu Corporation, Kyoto, Japan). cDNA was synthesized from 1 μg of RNA using the Superscript™ III First-Strand Synthesis System (Invitrogen), and amplified using specific primers for

Table 1. Primers used for reverse transcription–polymerase chain reaction (RT–PCR) amplification

Primers	Forward (5′–3′)	Reverse (3′–5′)	Predicted product size (bp)
hBD-2	CCA GCC ATC AGC CAT GAG GGT	GGA GCC CTT TCT GAA TCC GCA	256
GAPDH	GTC AAG GCT GAG AAC GGG AA	GCT TCA CCA CCT TCT TGA TG	613

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hBD-2, human β-defensin-2.

hBD-2 and a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in Table 1 and have been described previously (3).

Amplified products were analysed by electrophoresis on a 2% agarose gel in Tris-Acetate-EDTA (TAE) buffer (pH 8.3) (Nakarai Tesque, Inc., Kyoto, Japan), visualized by VersaDoc 5000 (Bio-Rad, Hercules, CA, USA) with ultraviolet (UV) illumination, making use of Syber Green (Invitrogen), and quantified by Analysis Software, Quantity One (Bio-Rad). The bands of each primer were analysed quantitatively relative to the bands of the housekeeping gene, GAPDH.

Detection of inflammatory cytokines in culture supernatants from HGEC

Culture supernatants from each culture of HGEC were analysed quantitatively by using interleukin-1β and interleukin-8 ELISA kits (Biosource International, Camarillo, CA, USA), according to the manufacturer's instructions.

Statistical analysis

All experiments were performed in triplicate. Levels of mRNA expression for hBD-2 were based upon semiquantitative RT–PCR evaluations. We analysed the data statistically, to determine the level of mRNA expression of hBD-2 and inflammatory cytokines in culture supernatants from HGEC, by using Student's *t*-test at the 0.05 level of significance.

Results

Under our experimental culture conditions, primary cultures of HGEC comprised > 99% keratinocytes, based on the immunohistochemical staining performed according to the report of Matsuyama *et al.* (9).

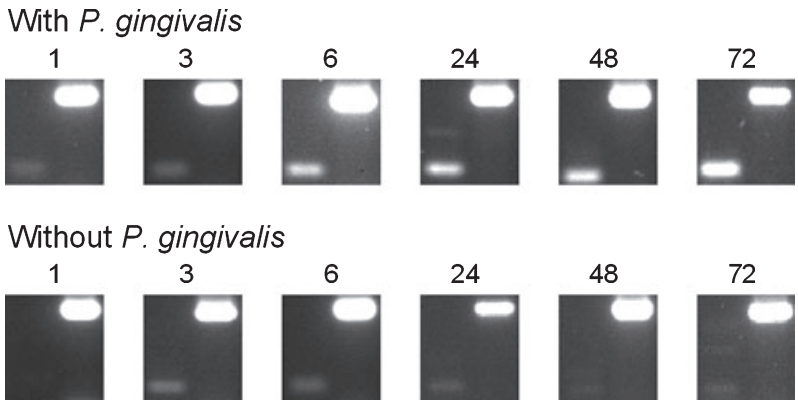

Fig. 1. Expression of human β-defensin-2 (hBD-2) mRNA, at various time points, in human gingival epithelial cells (HGEC) stimulated with or without *Porphyromonas gingivalis* compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The number shown above each panel represents the stimulation or culture time in hours. Total RNA was almost extracted from cultured cells in each well.

Expression of hBD-2 from HGEC stimulated with *P. gingivalis*

hBD-2 mRNA was expressed more strongly in HGEC stimulated with *P. gingivalis* than in the control HGEC (Fig. 1), and the level of expression clearly increased with time. All samples of GAPDH were also clearly visualized. The levels of hBD-2 mRNA in cells stimulated with *P. gingivalis* tended to increase with exposure time, but the level for control cells did not (Fig. 2). At 48 and 72 h, the hBD-2 mRNA level of the stimulated cells was significantly higher than that of the control cells, but no significant difference was observed at 1, 3, 6, or 24 h.

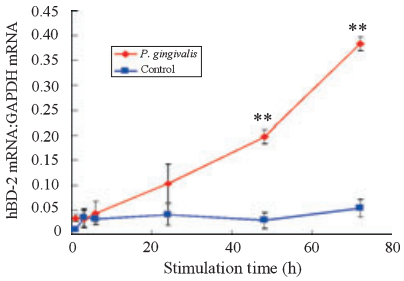

Fig. 2. The ratio of human β-defensin-2 (hBD-2) mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), based on semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) with visualization by VersaDoc 5000 (Bio-Rad). $^{**}p < 0.01$ compared with control cells at each time point.

Inflammatory cytokine production in culture supernatants from HGEC stimulated with *P. gingivalis*

ELISA for interleukin-8 and interleukin-1β, as representative inflammatory cytokines secreted by HGEC, revealed that the production of interleukin-8 was up-regulated in HGEC stimulated by *P. gingivalis* and in control cells, although to a much greater extent in stimulated HGEC than in control cells, and the difference was significant after 3 h ($p < 0.05$) (Fig. 3).

The production of interleukin-1β was also up-regulated in HGEC stimulated by *P. gingivalis* and in control cells, but to a lesser extent than interleukin-8 (Fig. 4). Moreover, although there was a significant difference between the groups at some time

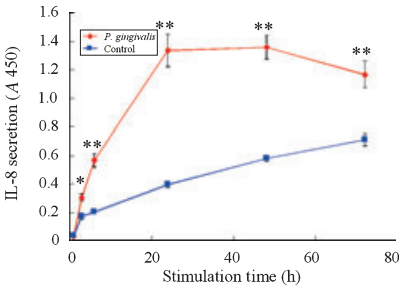

Fig. 3. The production of interleukin-8 (IL-8) in culture supernatants of human gingival epithelial cells (HGEC) stimulated with *Porphyromonas gingivalis* or control cells. The absorbance (*A*) was evaluated by enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed as described in the Material and methods. $*p < 0.05$, $**p < 0.01$ compared with control cells at each time point.

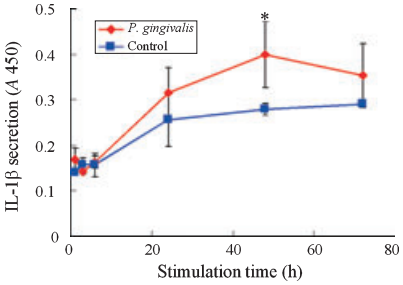

Fig. 4. The production of interleukin-1β (IL-1β) in culture supernatants of human gingival epithelial cells (HGEC) stimulated with *Porphyromonas gingivalis* or control cells. The absorbance (*A*) was evaluated by enzyme-linked immunosorbent assay (ELISA). Statistical analysis was performed as described in the Material and methods. $*p < 0.05$ compared with control cells at 48 h.

points, it was very small. In some samples at 48 h, the cells exposed to *P. gingivalis* had a significantly greater concentration of interleukin-1β than the control cells ($p < 0.05$).

Discussion

A significant difference in the expression of hBD-2 mRNA between HGEC stimulated with *P. gingivalis* and unstimulated cells was found after 48 h. Expression of hBD-2 mRNA did not increase in the control group (Fig. 2), but did increase in the *P. gingivalis*-stimulated group directly with stimulation time. There was also a significant difference in the concentration of interleukin-8 in the culture supernatant. Except for the 48-h time point, no significant difference was found for interleukin-1β between the two groups, although an increase with incubation time was observed, especially in the *P. gingivalis* stimulated-group (Fig. 4).

The pattern of expression of hBD-2 mRNA found, in the present study, for HGEC stimulated with *P. gingivalis* was similar to that reported for cells stimulated with *A. actinomycetemcomitans* (7). However, Krisanaprakornkit *et al.* (10) reported that cell wall extracts of *F. nucleatum* induced the expression of hBD-2 mRNA in HGEC, but cell wall extracts of *P. gingivalis* did not. The HGEC used in that study were obtained from gingival tissue specimens collected during the extraction of impacted third molars. These epithelial cells seem to be different from junctional epithelial cells, which comprise periodontal pocket epithelial tissue infected with anaerobic periodontal bacteria. Then, as the stimulating components, bacterial cell wall extracts were used, in contrast to the live *P. gingivalis* used in the present study. The results obtained in the present study differed from those reported by Krisanaprakornkit *et al.* probably as a result of the differences in the sites of origin of the cells and in the components used for stimulation. Marie *et al.* (11) reported that induction, by lipopolysaccharide, of hBD-2 in human tracheobronchial epithelial cells requires CD14, which may complex with a toll-like receptor to ultimately activate nuclear factor-κB. Cheryl *et al.* (12) and Thomas *et al.* (13) reported that tracheobronchial epithelial cells enhance the host defense by bacterial lipopeptide-stimulated induction of hBD-2 mRNA and protein via toll-like receptor 2 of the cells. Moreover, Puja *et al.* (14) reported that lipopolysaccharide and peptideoglycan stimulate β-defensin-2 promoter activation in a toll-like receptor 4- and toll-like receptor 2-dependent manner, respectively. As described above, periodontal pocket epithelial cells produce interleukin-8 and monocyte chemoattractant protein-1 via toll-like receptor 2 after stimulation with *P. gingivalis* (15). Therefore, CD14 and toll-like receptors in HGEC seemed to take part in the induction of hBD-2.

Expression of interleukin-8 in primary HGEC stimulated with *P. gingivalis* fimbriae and lipopolysaccharide was not significantly different from the expression of interleukin-8 in control cells at 24 h, but a difference was detected after stimulation with *P. gingivalis* sonic extracts (15). It has been reported that *A. actinomycetemcomitans* sonic extracts induce a significant increase in secretion of interleukin-8 after 4 h compared with the control, with a particularly marked increase between 12 h and 24 h, and a maximum level at 48 h (16). In the present study, significant differences were found between the *P. gingivalis*-stimulated group and the control group at 3 h in almost all samples, and marked increases were found up to 24 h. Stimulation with components of pathogenic periodontal bacteria seems to induce interleukin-8 elevation within 24 h as one of the initial inflammatory responses.

In the present study, we did not find a significant difference in the expression of interleukin-1β upon stimulation of HGEC with *P. gingivalis*, although it is reported that when both KB cells and cultured cells derived from periodontal pockets are stimulated with various pathogenic periodontal bacteria, the mRNA of a number of cytokines is expressed after 4 h, and stimulation with *P. gingivalis* 381, *P. gingivalis* 1743, and *P. gingivalis* DPG3 induces a significant elevation in the expression of interleukin-1β in both cell types (17). Live *A. actinomycetemcomitans* induces a dramatic increase of interleukin-1β with stimulation time (18). It has been reported that the production of interleukin-1β is significantly higher than the control value after 12 h, and that interleukin-1 is abundant in inflamed gingiva (16). Jandinski *et al.* reported that interleukin-1β-producing cells are present in numerous tissues from diseased sites and suggested that interleukin-1β, or the cells in these tissues, may be

associated with periodontal pathology (19). Stashenko *et al*. also reported that interleukin-1β levels in gingival tissues were related to the inflammatory status of periodontal tissues, specifically to changes in attachment levels and the presence of supragingival plaque or redness (20). These reports indicate that the production of interleukin-1β changes according to the inflammatory status of the periodontal tissues. In our study, we failed to find many significant differences. However, the production of interleukin-1β tended to increase with stimulation time, and that in the *P. gingivalis*-stimulated group was higher.

In the present study, we compared the expression of hBD-2 mRNA with the production of interleukin-8 and interleukin-1β. No relationship was found between hBD-2 mRNA expression and the concentration of interleukin-1β, as there was no significant variation of expression of interleukin-1β itself. However, we did find a close relationship between the expression of hBD-2 mRNA and the production of interleukin-8. Significant differences in the concentration of interleukin-8 were observed after 24 h in all samples and after 3 h in the majority of the samples stimulated with *P. gingivalis*, while the expression of hBD-2 mRNA showed a dramatic increase from 24 h to 48 h, and significant differences after 48 h. These results indicate that the production of interleukin-8 may influence the epithelial cells and is involved in the expression of hBD-2 mRNA by the mechanism mediated by cytokines look like an autocrine mechanism (21). Interleukin-8 can function as a migrating chemokine. Niyonsaba *et al*. (8) reported that hBD-2, being a potent chemoattractant for human neutrophils, as well as for interleukin-8, provides a novel mechanism for contributing to the host defense system by recruiting neutrophils to inflammation/infection sites. These experimental results, together with those of previous reports, suggest that the production of interleukin-8 is closely linked to the expression of hBD-2 mRNA involved in infection defense by the innate immune system.

These findings suggest that the expression of hBD-2 mRNA in HGEC was *P. gingivalis*-dependently induced according to stimulation time and is likely to be connected to the initial stages of the inflammatory response after infection with pathogenic periodontal bacteria, resulting ultimately in the migration of neutrophils to sites of infection. Ultrastructural confirmation of granular endoplasmic reticulum in cultured junctional epithelial cells suggests that a number of proteins are synthesized and a number of cytokines are released (22). Therefore, these effects may strengthen the innate immune function via an autocrine-like mechanism.

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