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Human β-defensin-3 up-regulates cyclooxygenase-2 expression and prostaglandin E_2 synthesis in human gingival fibroblasts

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Background and Objective: Oral epithelial cells express three antimicrobial peptide human β-defensins (hBDs) that have previously been demonstrated to exert proinflammatory effects on various immune cells. We wanted to examine whether hBDs could induce cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) synthesis in non-immune cells, such as human gingival fibroblasts.

Material and Methods: Cultured fibroblasts were treated with different concentrations of hBD-1, -2, -3 or interleukin-1β, as a positive control, for various times, in the presence or absence of NS-398, a specific COX-2 inhibitor. The levels of COX-1 and COX-2 mRNA expression were analyzed using RT-PCR and real-time PCR. Whole cell lysates were analyzed for COX-1 and COX-2 protein expression by western blotting. Cell-free culture supernatants were assayed for PGE_2 levels by ELISA. The lactate dehydrogenase assay was performed to determine the cytotoxicity of hBDs.

Results: Ten and 40 μg/mL of hBD-3 up-regulated COX-2 mRNA and protein expression, consistent with COX-2 up-regulation by interleukin-1β, whereas hBD-1 and hBD-2 did not. However, COX-1 mRNA and protein were constitutively expressed. The time-course study revealed that hBD-3 up-regulated COX-2 mRNA and protein expression at 6 and 12 h, respectively. Consistent with COX-2 up-regulation, 10 and 40 μg/mL of hBD-3 significantly increased PGE_2 levels in cell-free culture supernatants ($p < 0.05$), and this was inhibited by NS-398 in a dose-dependent manner. Neither of the hBD concentrations tested in this study was toxic to the cells.

Conclusion: These findings indicate that epithelial human β-defensin-3 functions as a proinflammatory mediator in controlling arachidonic acid metabolism in underlying fibroblasts.

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Oral stratified squamous epithelium expresses three human antimicrobial peptides of the β-defensin family, human β-defensin (hBD)-1, -2 and -3, as part of its innate immunity (1). Previous *in vitro* studies have shown antimicrobial activities of hBD-2 and hBD-3 in the concentration range of micrograms per millilitre against two critical periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, formerly

known as *Actinobacillus actinomycetemcomitans* (2–5). This suggests their potential role as antimicrobial agents for periodontal disease. Moreover, differential expression of hBD-1 and hBD-2 is reported in specific types of periodontal disease (6). In particular, significantly higher hBD-1 expression has been reported in chronic than in aggressive periodontitis, while significantly higher hBD-2 expression has been reported in aggressive than in chronic periodontitis. In addition to their antimicrobial activities, it is now recognized that these β-defensins can function as immunomodulators. For example, hBD-1 and hBD-2 serve as a link between innate and acquired immunity by acting as chemo-attractants for immature dendritic cells and T lymphocytes (7), and as immunomodulators in mast cells (8). Moreover, both hBD-2 and hBD-3 stimulate primary human keratinocytes to produce various proinflammatory cytokines and chemokines (9,10), and hBD-2 activates many inducible genes involved in immune and defense responses in oral epithelial cells (11) in an autocrine fashion. Likewise, it is possible that these oral epithelial human β-defensins exert their immunomodulatory activities in another cell type of periodontium, i.e. human gingival fibroblasts, in a paracrine fashion. In fact, it has been shown that hBD-2 and hBD-3 significantly induce cell proliferation in primary human gingival fibroblasts (12).

It has been demonstrated that prostaglandin E_2 (PGE_2) levels in gingival crevicular fluid are significantly higher in patients with chronic periodontitis than in normal patients (13–16) and in cell-free culture supernatants collected from cultured human periodontal ligament and gingival fibroblasts stimulated with proinflammatory stimuli through induced cyclooxygenase-2 (COX-2) expression than in those collected from non-stimulated cells (17–19). This may imply that COX-2 plays a crucial role in PGE_2 production in periodontal disease. Prostaglandin E_2 can be synthesized by both cyclooxygenase-1 (COX-1) and COX-2. Generally, COX-1 is constitutively expressed in many tissues and supports prostanoid synthesis, regulated for maintaining organ and tissue homeostasis, whereas COX-2 is induced after stimulation with proinflammatory molecules and is up-regulated during tissue inflammation, especially in periodontal disease. Since both COX-2 and PGE_2, two critical inflammatory markers in the pathogenesis of periodontal disease (20–22), are up-regulated by interleukin-1β (IL-1β) in cultured human gingival fibroblasts (19), and hBDs are implicated in both innate immunity and inflammation (23), we, therefore, wanted to examine the proinflammatory effects of hBDs on gingival fibroblasts. The objective of this study was to determine the effects of hBDs on up-regulating expression of COX-2 and synthesis of PGE_2 in gingival fibroblasts. It was found that only hBD-3 up-regulated COX-2 mRNA and protein expression, resulting in raised PGE_2 levels in cell-free culture supernatants. This suggests that oral epithelial hBD-3 plays an important role as another proinflammatory mediator in controlling arachidonic acid metabolism in underlying gingival fibroblasts, in addition to its known antimicrobial activities.

Material and methods

Materials

Human β-defensin-1, -2 and -3 were purchased from the Peptide Institute, Inc., Osaka, Japan. The polyclonal antibodies against human COX-1, COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Interleukin-1β was purchased from R&D Systems, Minneapolis, MN, USA. A specific inhibitor of COX-2 activity, NS-398, was obtained from Cayman Chemical, Ann Arbor, MI, USA.

Cell culture

Primary human gingival fibroblasts were isolated from gingival biopsies overlying impacted third molars, collected from healthy patients undergoing extraction of impacted teeth at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chiang Mai University, as described previously (24). Briefly, the gingival biopsies were cut into small pieces with dimensions of 2 mm wide and 2 mm long. The biopsies were then placed on a 60 mm culture dish (Nunc™, Roskilde, Denmark) and cultured in Dulbecco's modified Eagle's medium (Invitrogen™, Grand Island, NY, USA), containing 10% fetal bovine serum (Invitrogen™) and 1% penicillin/streptomycin (Invitrogen™), until the fibroblasts expanded and reached confluence. Subsequently, cells were further passaged to expand their number, and cell lines from three different donors ($n = 3$) from the third to fifth passages were used throughout this study. The research protocol had been approved by the Local Human Experimentation Committee (#1/2009), and written informed consent was obtained from the patients. After confluence, cells (2×10^5 per well) were starved of serum for 6 h prior to treatment with various concentrations of hBD-1, -2, -3 or IL-1β, as a positive control, for various times, in the presence or absence of NS-398.

Isolation of total RNA and RT-PCR

Total RNA was harvested according to the manufacturer's protocols, using an Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA, USA). The RT-PCR protocol has been described previously (25). Briefly, 3 μg of total RNA were used for synthesis of cDNA using a SuperScript™ First-Strand cDNA System (Fermentas, Hanover, MD, USA). The PCR primers for COX-1 were 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ (forward) and 5′-GTGCATCAACACAGGCGCCTCTTC-3′ (reverse); and for COX-2 5′-TTCAAATGAGATTGTGGGAAAAT-3′ (forward) and 5′-AGATGNATCTCTGCCTGAGTATCTT-3′ (reverse). The PCR amplification for COX-1 and COX-2 consisted of 28 and 35 cycles, respectively, of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with an initial denaturation for 3 min at 94°C. The PCR primers for GAPDH were 5′-ACCACAGTCCATGCCATCACTGC-3′

(forward) and 5′-TCCACCACCCTG TTGCTGTAGC-3′ (reverse). The PCR amplification for GAPDH consisted of 20 cycles of 95°C for 45 s, 60°C for 1 min and 72°C for 2 min. The PCR products were resolved on 1.2% agarose gel, and photographs were taken by a CCD camera, attached to the Chemidoc XRS documentation system (Bio-Rad Laboratories). The predicted sizes of PCR product for COX-1, COX-2 and GAPDH were 304, 305, and 452 bp, respectively. The results from sequencing reactions of PCR products at Macrogen Inc., Seoul, Korea, confirmed 100% identities with the complete coding sequences of COX-1 mRNA (Genbank accession no. NM080591) and COX-2 mRNA (Genbank accession no. NM000963).

Real-time PCR

The real-time PCR was performed using 5% (vol/vol) of cDNA from the reverse transcription, 1 μL of each 10 mM primer, and 10 μL of the Biotools QUANTIMIX EASY SYG reagents (Biotools, B & M Labs, Madrid, Spain) in the Rotor-Gene™ 6000 (Corbett Research, Sydney, NSW, Australia). The real-time PCR amplification in a total volume of 20 μL consisted of 40 cycles of 95°C for 45 s, 60°C for 1 min and 72°C for 2 min for COX-1, COX-2 and GAPDH. The ratio of COX-1 or COX-2 relative to GAPDH expression in each sample was calculated from three separate experiments. The median value (fold) of COX-1 or COX-2 mRNA induction was determined by comparing the ratio of COX-1 or COX-2 relative to GAPDH expression of an hBD-treated sample with that of an untreated control sample.

Western blot analysis

Whole cell lysates of control and treated human gingival fibroblasts were extracted in RIPA buffer (26). Forty micrograms of cell lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked, reacted with primary antibody against COX-1, COX-2 or GAPDH at 1:400 dilution, and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilution. LumiGLO Reserve Chemiluminescence (KPL, Gaithersburg, MD, USA) was used as a substrate, and the signal was captured with a CCD camera, attached to the ChemiDoc XRS.

Prostaglandin E_2 assay

Cell-free culture supernatants were collected and analyzed for PGE_2 levels using the commercially available ELISA kit (R&D Systems). Briefly, 150 μL of the standard included in the kit or of the cell-free culture supernatant samples and 50 μL of primary antibody solution were added to each well. Subsequently, 50 μL of PGE_2 conjugate was added and incubated for 2 h at ambient temperature. The well was then washed, and 200 μL of substrate solution was added and incubated for 30 min under light protection. The reaction was stopped, and the developing color was determined by optical density using the Titertek Multiskan M340 multiplate reader (ICN Flow, Costa Mesa, CA, USA), set to 450 nm within 30 min.

Lactate dehydrogenase (LDH) assay

To determine the cytotoxicity of various doses of hBDs on human gingival fibroblasts, an LDH assay was performed. An extracellular release of LDH is a common marker for cell death. Therefore, cell-free culture supernatants were collected from cells that were incubated with various doses of hBD-1, -2, -3 or 5 mM of H_2O_2, as a positive control, for 24 h. The LDH mixture solution, containing 2 mM of pyruvic acid (Sigma-Aldrich, St Louis, MO, USA) and 0.3 mM of β-nicotinamide adenine dinucleotide (Sigma-Aldrich) in phosphate buffer solution (50 mM of $NaHPO_4$ and 8 mM of KH_2PO_4, pH 7.4), was added to 100 μL of the culture supernatants or to a blank control at 37°C for 30 min. Subsequently, 1 mL of the color reagent [2,4-dinitrophenylhydrazine (Sigma-Aldrich)] was added and incubated at room temperature for 20 min. The reaction was stopped by addition of 0.4 M of NaOH, and the developing color was determined as an optical density using the Titertek Multiskan M340 multiplate reader, set to 450 nm.

Statistical analyses

The differences in terms of the fold of COX-2 mRNA induction and the PGE_2 levels shown in Fig. 4 and in Tables 1 and 2 were tested by the Kruskal–Wallis *H*-test and the Mann–Whitney *U*-test at $p < 0.05$. The differences in the arbitrary LDH units/mL that indicated cell death, shown in Fig. 3, between untreated control and hBD-treated or H_2O_2-treated samples, were tested by Student's paired *t*-test at $p < 0.01$.

Results

Human β-defensin-3 up-regulated COX-2 expression in both dose- and time-dependent manners

To determine the proinflammatory effects of hBDs, human gingival fibroblasts were treated with various doses of hBDs for different times. Cells were harvested for analyses of COX-2 mRNA and protein expression. The results showed that hBD-3 up-regulated COX-2 mRNA in a dose-dependent manner (Fig. 1A). Similarly, IL-1β, as a positive control for cell stimulation, induced COX-2 mRNA expression at concentrations as low as 1 ng/mL (Fig. 1B). However, neither hBD-1 nor hBD-2 at any dose induced COX-2 mRNA (Fig. 1A). Consistent with the findings of other studies (27,28), COX-1 mRNA was constitutively expressed in human gingival fibroblasts (Fig. 1). The real-time PCR analysis showed a significant COX-2 mRNA induction by 10 and 40 μg/mL of hBD-3 ($p < 0.05$; Table 1). The time-course study showed an obvious COX-2 mRNA induction after human gingival fibroblasts were treated with 10 μg/mL of hBD-3 for 6 h (Fig. 1C), whereas COX-2 protein induction

Table 1. Human β-defensin-3 treatment resulted in significant cyclooxygenase-2 (COX-2) mRNA up-regulation and elevated prostaglandin E_2 (PGE_2) levels in cell-free culture supernatants in a dose-dependent fashion

Concentration (μg/mL)	Median fold (range) of COX-2/ GAPDH ratio	Median PGE_2 concentration (range) (pg/mL)
Control	1.00	36.40 (34.49–38.32)
hBD-1 0.3	1.16 (1.10–1.21)	35.91 (33.74–38.08)
hBD-1 1.0	1.28 (1.20–1.36)	39.22 (36.28–42.16)
hBD-1 3.0	1.21 (1.20–1.22)	37.42 (34.99–39.85)
hBD-1 10.0	1.48 (1.40–1.56)	37.35 (34.96–39.73)
hBD-1 40.0	1.20 (1.20–1.20)	39.61 (35.43–43.78)
hBD-2 0.3	1.03 (1.00–1.06)	38.30 (35.70–40.89)
hBD-2 1.0	1.18 (1.20–1.16)	37.58 (35.00–40.16)
hBD-2 3.0	1.61 (1.00–2.22)	39.64 (34.86–44.42)
hBD-2 10.0	1.21 (1.20–1.21)	40.51 (38.37–42.66)
hBD-2 40.0	1.01 (0.82–1.20)	36.82 (32.94–40.69)
hBD-3 0.3	3.02 (2.58–3.46)	21.33 (20.00–23.00)
hBD-3 1.0	3.35 (2.68–4.03)	35.55 (22.64–48.31)
hBD-3 3.0	6.20 (4.72–7.67)	53.16 (48.31–58.00)
hBD-3 10.0	24.14* (23.26–25.01)	260.59* (260.56–266.03)
hBD-3 40.0	259.00* (233.94–284.05)	1934.00* (1824.20–2048.10)

The real-time PCR assay was performed using the samples from Fig. 1A to analyze the ratios of COX-2 relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, and these ratios were compared between untreated control and hBD-treated cells and expressed as median folds of induction. The cell-free culture supernatants from Fig. 1A were collected and analyzed for PGE_2 concentration by ELISA.
*Statistically significant difference from control untreated cells at $p < 0.05$.

Table 2. The kinetic study showed that hBD-3 significantly up-regulated COX-2 mRNA expression at 6 h and PGE_2 synthesis in cell-free culture supernatants at 12 h

hBD-3 (10 μg/mL) treatment time (h)	Median fold (range) of COX-1/ GAPDH ratio	Median fold (range) of COX-2/ GAPDH ratio	Median PGE_2 concentration (range) (pg/mL)
0	1.00	1.00	35.16 (33.00–37.32)
1	0.96 (0.93–0.99)	1.80 (1.72–1.88)	35.07 (34.44–35.70)
3	0.90 (0.85–0.98)	1.71 (1.66–1.77)	36.34 (34.53–38.15)
6	0.96 (0.88–1.03)	5.25* (4.83–5.66)	43.16 (38.77–47.56)
12	0.92 (0.79–1.02)	15.77* (15.54–16.00)	74.23* (66.57–81.88)
24	0.98 (0.89–1.07)	14.90* (14.34–15.45)	123.93* (122.91–124.95)

The real-time PCR assay was performed using the samples from Fig. 1C to analyze the ratios of either COX-1 or COX-2 relative to GAPDH expression, and these ratios were compared between untreated control and hBD-3-treated cells and expressed as median folds of induction. The cell-free culture supernatants from Figs 1C and 2B were collected and analyzed for PGE_2 concentration by ELISA. Note constitutive COX-1 mRNA expression.
*Statistically significant difference from control cells (0 h) at $p < 0.05$.

occurred at 12 h (Fig. 2B), while COX-1 protein expression remained unchanged (Fig. 2). Consistent with COX-2 mRNA induction, COX-2 protein expression was remarkably up-regulated by 10 and 40 μg/mL of hBD-3 (Fig. 2A). The COX-2 mRNA induction was significant at 6 h ($p < 0.05$), and the induction reached a maximal level at 12 h (Table 2).

Human β-defensin-3 elevated levels of PGE_2

To examine the synthesis of PGE_2, cell-free culture supernatants from human gingival fibroblasts, treated with various concentrations of hBDs for different times, were analyzed by ELISA. In agreement with COX-2 induction, the PGE_2 levels were significantly elevated in cell-free culture supernatants collected from cells treated with 10 and 40 μg/mL of hBD-3 ($p < 0.05$, $r = 0.998$; Table 1) and with 10 μg/mL of hBD-3 at 12 and 24 h ($p < 0.05$, $r = 0.861$; Table 2).

No cytotoxicity of human β-defensins to human gingival fibroblasts

To determine the cell death after being treated with each hBD, human gingival fibroblasts were plated, and then treated with either various doses (up to 40 μg/mL) of each hBD or 5 mM of H_2O_2, as a positive control for cell toxicity, for 24 h. It was demonstrated that there was no significant difference in terms of the values in LDH units/mL between hBD-treated and untreated control cells ($p > 0.05$; Fig. 3). In contrast, these values from H_2O_2-treated cells were significantly higher than those from untreated control cells ($p < 0.01$; Fig. 3). This indicates that neither of the hBD doses tested in this study was toxic to the cells, whereas treatment with 5 mM of H_2O_2 resulted in cell death.

Elevated PGE_2 synthesis stimulated by hBD-3 was via COX-2 induction

To examine whether elevated PGE_2 levels in cell-free culture supernatants collected from human gingival fibroblasts treated with hBD-3 resulted from COX-2 induction, cells were pretreated with 0.01, 0.1 or 1 μM of NS-398, a specific inhibitor of COX-2 activity (28), 30 min prior to treatment with either 10 μg/mL of hBD-3 or 10 ng/mL of IL-1β, as a positive control. As expected, the elevated levels of PGE_2 resulting from stimulation with either hBD-3 or IL-1β were significantly decreased by pretreatment with NS-398 in a dose-dependent manner ($p = 0.008$ for hBD-3; $p = 0.002$ for IL-1β; Fig. 4A and B, respectively). Taken together, these results suggest that oral epithelial hBD-3 can exert its proinflammatory and paracrine effects by up-regulating COX-2 expression and PGE_2 synthesis through induced COX-2 expression in underlying gingival fibroblasts.

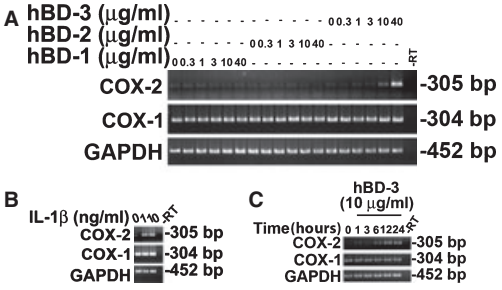

Fig. 1. Up-regulation of COX-2 mRNA by hBD-3 in human gingival fibroblasts. (A) Cells were treated with hBD-1, -2 or -3 at 0.3, 1, 3, 10 and 40 μg/mL for 18 h, or left untreated as a control. (B) COX-2 mRNA induction by IL-1β. Cells were incubated with 1 or 10 ng/mL of IL-1β for 18 h, or left untreated as a control. (C) Cells were treated with 10 μg/mL of hBD-3 for 1, 3, 6, 12 and 24 h, or left untreated as a control. After incubation, total RNA was harvested and analyzed for COX-1, COX-2 and GAPDH mRNA expression by RT-PCR. The results shown are representative of three independent experiments.

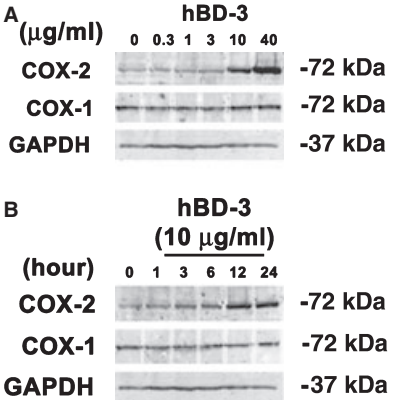

Fig. 2. Induction of COX-2 protein by hBD-3 in human gingival fibroblasts. (A) Cells were incubated with 0.3, 1, 3, 10 or 40 μg/mL of hBD-3 for 18 h, , or left untreated as a control. (B) Cells were incubated with 10 μg/mL of hBD-3 for 1, 3, 6, 12 and 24 h, or left untreated as a control. Whole cell lysates were extracted, and expression of COX-1, COX-2 and GAPDH protein were analyzed by western blotting, as described in the Material and Methods section. The results shown are representative of three independent experiments.

Discussion

In the present study, we have shown the effects of hBD-3 on induction of COX-2 mRNA and protein expression, and consistently elevated PGE_2 levels without discernible or measurable cytotoxicity. In addition, the elevation of PGE_2 levels by hBD-3 treatment was inhibited by pretreatment with NS-398, suggesting that production of PGE_2 was up-regulated via COX-2 induction. The facts that elevated PGE_2 concentrations have been shown in inflamed gingival tissue (13) and in gingival crevicular fluid of patients with chronic periodontitis (15) and are associated with the pathogenesis of periodontal disease through induced COX-2 expression (21) suggest a critical role for hBD-3 as another proinflammatory mediator in periodontal disease. The observation that only hBD-3, but not hBD-1 or hBD-2, induces COX-2 expression may not simply be explained by stress on the fibroblast cell membrane, induced by cationic peptides, since these three β-defensin peptides have similar molecular structures and net positive charges (29). Therefore, a more specific mechanism of controlling COX-2 up-regulation by hBD-3 is likely to occur. Further investigations are required to elucidate specific receptor(s) and signaling pathway(s), which may include p38 MAPK, which has been shown to be involved in COX-2 induction by *Streptococcus pneumoniae* (30), and nuclear factor-κB, which is implicated in IL-1β-induced COX-2 expression (31).

Apart from the fact that in our study we used the same hBD concentrations, i.e. in low microgram per millilitre levels, that were used in others (9,10), it is anticipated that the concentrations of each hBD used in our study (ranging from 0.3 to 40 μg/mL), which were not toxic to gingival fibroblasts, are appropriate for evaluating the physiological roles of these β-defensins *in vivo*. This is because the amounts of hBD-2 and hBD-3 *in vitro* are estimated to be approximately 16 μM (~80 μg/mL) in IL-1α-stimulated organotypic epidermal cultured cells (32) and 34 μM (~170 μg/mL) in *Staphylococcus aureus*-stimulated normal epidermis (33), respectively. *In vivo*, especially in the oral cavity, the median levels of hBD-3 have been shown to vary within the microgram per millilitre range in the whole saliva of 149 middle school children, most of which were Hispanic, with some Native Americans and Caucasians (34). Consequently, low microgram per millilitre concentrations of these β-defensins are likely to exist within oral mucosal tissue during the pathophysiological processes of oral inflammatory disorders, although the

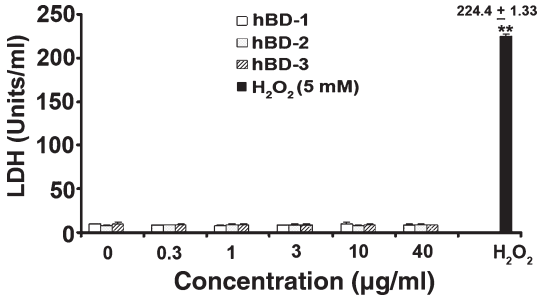

Fig. 3. No toxicity was found in human gingival fibroblasts treated with human β-defensins by a lactate dehydrogenase (LDH) assay. Cells (at a cell density of 1×10^4) were treated with 0.3, 1, 3, 10 and 40 μg/mL of hBD-1, -2 or -3 for 24 h, and the LDH release, measured as LDH units/mL, in cell-free culture supernatants was analyzed and then compared between untreated control cells and H_2O_2-treated or hBD-treated cells. Data are expressed as means ± SD of ten independent experiments, and the significant differences were tested by Student's paired *t*-test at $^{**}p < 0.01$.

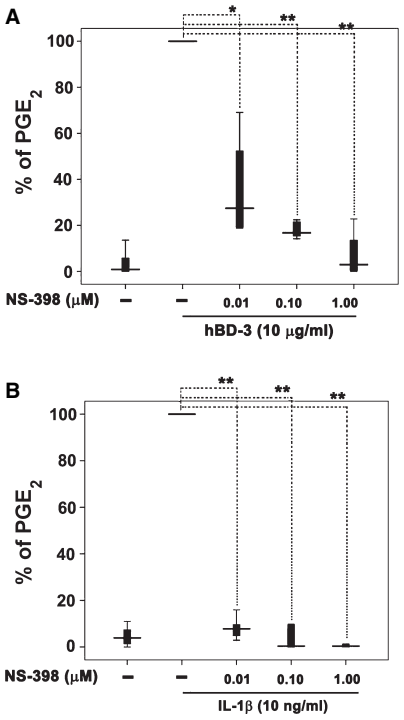

Fig. 4. Inhibition of PGE_2 synthesis in human gingival fibroblasts pretreated with a selective COX-2 inhibitor, NS-398. Cells were treated either with 10 μg/mL of hBD-3 (A) or with 10 ng/mL of IL-1β (B), in the presence or absence of 0.01, 0.10 or 1.00 μM of NS-398 for 18 h. Cell-free culture supernatants were collected and assayed for PGE_2 levels by ELISA, as described in the Material and methods section. The PGE_2 levels in all samples are expressed as the percentages of PGE_2 levels in inhibitor-treated samples compared with either the hBD-3- or the IL-1β-treated samples, set at 100%. The results shown are representative of three independent experiments. *Statistically significant difference at $p < 0.05$; **statistically significant difference at $p < 0.01$.

amounts of each β-defensin have not yet been precisely quantified.

The significance of this study is to show the proinflammatory effects of hBD-3, an inducible antimicrobial peptide in the β-defensin family, derived from oral epithelial cells, on controlling arachidonic acid metabolism in non-immune cells, particularly gingival fibroblasts, besides its known immunomodulation in various types of immune cells. The metabolism of arachidonic acid follows multiple and interrelated pathways, leading to generation of a wide variety of biologically active substances, such as prostanoids, leukotrienes, epoxyeicosatrienoic acid, etc., which eventually lead to pain and inflammation (35). The mechanisms of pain and inflammation should be viewed as a complex and interrelated network, and various enzymes and pathways in arachidonic acid metabolism are implicated. It is, therefore, necessary to further investigate the proinflammatory effects of hBD-3 on the expression of other enzymes involved in arachidonic acid metabolism and on the levels of other active metabolites, in addition to COX-2 and PGE_2. It is probable that hBD-3 also affects expression of a number of phospholipase A_2 enzymes that control the arachidonic acid levels in fibroblasts.

The findings from a previous study (36) that demonstrated the localization of hBD-3 in the basal layer of gingival epithelium are consistent with our results, which suggest potential cross-talk between gingival epithelial cells and underlying fibroblasts via hBD-3 acting in a paracrine fashion. Furthermore, it has been shown that increased PGE_2 levels can, in turn, affect β-defensin expression in gingival epithelial cells (37), emphasizing the roles of these periodontal cells in enhancement of inflammation. In addition, it is interesting to examine the proinflammatory effects of hBDs on arachidonic acid metabolism in other non-immune cells that reside in gingival tissue, such as epithelial cells, periodontal ligament fibroblasts, endothelial cells, etc. In fact, it has recently been shown that hBD-2 stimulates migration and proliferation of human endothelial cells, suggesting its role in proangiogenic activity (38). In summary, oral epithelium-derived human β-defensin-3 can function as a proinflammatory mediator by regulating COX-2 expression and PGE_2 synthesis in underlying gingival fibroblasts in a paracrine fashion, suggesting its critical role in inflammatory periodontal disease.

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References

1. Abiko Y, Saitoh M, Nishimura M, Yamazaki M, Sawamura D, Kaku T. Role of beta-defensins in oral epithelial health and disease. *Med Mol Morphol* 2007; **40:**179–184.
2. Guthmiller JM, Vargas KG, Srikantha R *et al.* Susceptibilities of oral bacteria and yeast to mammalian cathelicidins. *Antimicrob Agents Chemother* 2001;**45:**3216–3219.
3. Maisetta G, Batoni G, Esin S *et al.* Activity of human beta-defensin 3 alone or combined with other antimicrobial agents against oral bacteria. *Antimicrob Agents Chemother* 2003;**47:**3349–3351.
4. Mineshiba F, Takashiba S, Mineshiba J, Matsuura K, Kokeguchi S, Murayama Y. Antibacterial activity of synthetic human β-defensin-2 against periodontal bacteria. *J Int Acad Periodontol* 2003;**5:**35–40.
5. Joly S, Maze C, McCray PB Jr, Guthmiller JM. Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. *J Clin Microbiol* 2004;**42:**1024–1029.
6. Vardar-Sengul S, Demirci T, Sen BH, Erkizan V, Kurulgan E, Baylas H. Human beta defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases. *J Periodontal Res* 2007;**42:**429–437.
7. Yang D, Chertov O, Bykovskaia SN *et al.* Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* 1999;**286:**525–528.
8. Niyonsaba F, Someya A, Hirata M, Ogawa H, Nagaoka I. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur J Immunol* 2001; **31:**1066–1075.
9. Niyonsaba F, Ushio H, Nagaoka I, Okumura K, Ogawa H. The human beta-

defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes. *J Immunol* 2005; **175:**1776–1784.
10. Niyonsaba F, Ushio H, Nakano N *et al*. Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J Invest Dermatol* 2007;**127:**594–604.
11. Yin L, Dale BA. Activation of protective responses in oral epithelial cells by *Fusobacterium nucleatum* and human beta-defensin-2. *J Med Microbiol* 2007;**56:** 976–987.
12. Nishimura M, Abiko Y, Kurashige Y *et al*. Effect of defensin peptides on eukaryotic cells: primary epithelial cells, fibroblasts and squamous cell carcinoma cell lines. *J Dermatol Sci* 2004;**36:**87–95.
13. Mendieta CF, Reeve CM, Romero JC. Biosynthesis of prostaglandins in gingiva of patients with chronic periodontitis. *J Periodontol* 1985;**56:**44–47.
14. ElAttar TM. Prostaglandin E2 in human gingiva in health and disease and its stimulation by female sex steroids. *Prostaglandins* 1976;**11:**331–341.
15. Offenbacher S, Odle BM, Van Dyke TE. The use of crevicular fluid prostaglandin E2 levels as a predictor of periodontal attachment loss. *J Periodontal Res* 1986; **21:**101–112.
16. Tsai CC, Hong YC, Chen CC, Wu YM. Measurement of prostaglandin E2 and leukotriene B4 in the gingival crevicular fluid. *J Dent* 1998;**26:**97–103.
17. Noguchi K, Shitashige M, Ishikawa I. Involvement of cyclooxygenase-2 in interleukin-1alpha-induced prostaglandin production by human periodontal ligament cells. *J Periodontol* 1999;**70:**902–908.
18. Bando Y, Noguchi K, Kobayashi H, Yoshida N, Ishikawa I, Izumi Y. Cyclooxygenase-2-derived prostaglandin E2 is involved in vascular endothelial growth factor production in interleukin-1alpha-stimulated human periodontal ligament cells. *J Periodontal Res* 2009;**44:**395–401.
19. Yucel-Lindberg T, Ahola H, Nilsson S, Carlstedt-Duke J, Modeer T. Interleukin-1 beta induces expression of cyclooxygenase-2 mRNA in human gingival fibroblasts. *Inflammation* 1995;**19:**549–560.
20. Offenbacher S, Heasman PA, Collins JG. Modulation of host PGE2 secretion as a determinant of periodontal disease expression. *J Periodontol* 1993;**64:**432–444.
21. Zhang F, Engebretson SP, Morton RS *et al*. The overexpression of cyclo-oxygenase-2 in chronic periodontitis. *J Am Dent Assoc* 2003;**134:**861–867.
22. Kardesler L, Buduneli N, Biyikoglu B, Cetinkalp S, Kutukculer N. Gingival crevicular fluid PGE2, IL-1beta, t-PA, PAI-2 levels in type 2 diabetes and relationship with periodontal disease. *Clin Biochem* 2008;**41:**863–868.
23. Weinberg A, Krisanaprakornkit S, Dale BA. Epithelial antimicrobial peptides: review and significance for oral applications. *Crit Rev Oral Biol Med* 1998;**9:**399–414.
24. Krisanaprakornkit S, Kimball JR, Weinberg A, Darveau RP, Bainbridge BW, Dale BA. Inducible expression of human beta-defensin 2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun* 2000; **68:**2907–2915.
25. Krisanaprakornkit S, Chotjumlong P, Kongtawelert P, Reutrakul V. Involvement of phospholipase D in regulating expression of anti-microbial peptide human beta-defensin-2. *Int Immunol* 2008; **20:**21–29.
26. Krisanaprakornkit S, Kimball JR, Dale BA. Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family. *J Immunol* 2002; **168:**316–324.
27. Yucel-Lindberg T, Ahola H, Carlstedt-Duke J, Modeer T. Involvement of tyrosine kinases on cyclooxygenase expression and prostaglandin E2 production in human gingival fibroblasts stimulated with interleukin-1beta and epidermal growth factor. *Biochem Biophys Res Commun* 1999;**257:**528–532.
28. Noguchi K, Iwasaki K, Shitashige M, Endo H, Kondo H, Ishikawa I. Cyclooxygenase-2-dependent prostaglandin E2 down-regulates intercellular adhesion molecule-1 expression via EP2/EP4 receptors in interleukin-1beta-stimulated human gingival fibroblasts. *J Dent Res* 2000;**79:**1955–1961.
29. Taylor K, Barran PE, Dorin JR. Structure-activity relationships in beta-defensin peptides. *Biopolymers* 2008;**90:**1–7.
30. N'Guessan PD, Hippenstiel S, Etouem MO *et al*. *Streptococcus pneumoniae* induced p38 MAPK- and NF-kappaB-dependent COX-2 expression in human lung epithelium. *Am J Physiol Lung Cell Mol Physiol* 2006;**290:**L1131–L1138.
31. Nakao S, Ogata Y, Shimizu-Sasaki E, Yamazaki M, Furuyama S, Sugiya H. Activation of NFkappaB is necessary for IL-1beta-induced cyclooxygenase-2 (COX-2) expression in human gingival fibroblasts. *Mol Cell Biochem* 2000;**209:** 113–118.
32. Liu AY, Destoumieux D, Wong AV *et al*. Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J Invest Dermatol* 2002;**118:**275–281.
33. Kisich KO, Carspecken CW, Fieve S, Boguniewicz M, Leung DY. Defective killing of *Staphylococcus aureus* in atopic dermatitis is associated with reduced mobilization of human beta-defensin-3. *J Allergy Clin Immunol* 2008;**122:**62–68.
34. Tao R, Jurevic RJ, Coulton KK *et al*. Salivary antimicrobial peptide expression and dental caries experience in children. *Antimicrob Agents Chemother* 2005; **49:**3883–3888.
35. Smith HS. Arachidonic acid pathways in nociception. *J Support Oncol* 2006;**4:**277–287.
36. Lu Q, Samaranayake LP, Darveau RP, Jin L. Expression of human beta-defensin-3 in gingival epithelia. *J Periodontal Res* 2005;**40:**474–481.
37. Noguchi T, Shiba H, Komatsuzawa H *et al*. Syntheses of prostaglandin E2 and E-cadherin and gene expression of beta-defensin-2 by human gingival epithelial cells in response to *Actinobacillus actinomycetemcomitans*. *Inflammation* 2003;**27:** 341–349.
38. Baroni A, Donnarumma G, Paoletti I *et al*. Antimicrobial human beta-defensin-2 stimulates migration, proliferation and tube formation of human umbilical vein endothelial cells. *Peptides* 2009;**30:**267–272.