Inducible Expression of Human β-Defensin 2 by Fusobacterium nucleatum in Oral Epithelial Cells: Multiple Signaling Pathways and Role of Commensal Bacteria in Innate Immunity and the Epithelial Barrier

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Human gingival epithelial cells (HGE) express two antimicrobial peptides of the β-defensin family, human β-defensin 1 (hBD-1) and hBD-2, as well as cytokines and chemokines that contribute to innate immunity. In the present study, the expression and transcriptional regulation of hBD-2 was examined. HBD-2 mRNA was induced by cell wall extract of Fusobacterium nucleatum, an oral commensal microorganism, but not by that of Porphyromonas gingivalis, a periodontal pathogen. HBD-2 mRNA was also induced by the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) and phorbol myristate acetate (PMA), an epithelial cell activator. HBD-2 mRNA was also expressed in 14 of 15 noninflamed gingival tissue samples. HBD-2 peptide was detected by immunofluorescence in HGE stimulated with F. nucleatum cell wall, consistent with induction of the mRNA by this stimulant. Kinetic analysis indicates involvement of multiple distinct signaling pathways in the regulation of hBD-2 mRNA; TNF-α and F. nucleatum cell wall induced hBD-2 mRNA rapidly (2 to 4 h), while PMA stimulation was slower (~10 h). In contrast, each stimulant induced interleukin 8 (IL-8) within 1 h. The role of TNF-α as an intermediary in F. nucleatum signaling was ruled out by addition of anti-TNF-α that did not inhibit hBD-2 induction. However, inhibitor studies show that F. nucleatum stimulation of hBD-2 mRNA requires both new gene transcription and new protein synthesis. Bacterial lipopolysaccharides isolated from Escherichia coli and F. nucleatum were poor stimulants of hBD-2, although they up-regulated IL-8 mRNA. Collectively, our findings show inducible expression of hBD-2 mRNA via multiple pathways in HGE in a pattern that is distinct from that of IL-8 expression. We suggest that different aspects of innate immune responses are differentially regulated and that commensal organisms have a role in stimulating mucosal epithelial cells in maintaining the barrier that contributes to homeostasis and host defense.

Mucosal epithelial cells play an integral role in innate immune defense by sensing signals from the external environment, generating various molecules to affect growth, development, and function of other cells, and maintaining the balance between health and disease (23). Mucosal epithelial cells express antimicrobial peptides, including the β-defensins human β-defensin 1 (hBD-1) and hBD-2, as well as chemokines that attract monocytes and neutrophils and cytokines that activate the adaptive immune system (23). It is now recognized that the antimicrobial peptide hBD-2 also stimulates antigen-presenting dendritic cells that signal the adaptive immune system (51), in addition to its antimicrobial activity. Therefore, characterization of β-defensin regulation is essential for understanding the role of these peptides in protecting the host by activating both innate and adaptive immune systems and in contributing to the epithelial barrier to inflammatory disease processes.

It is now widely recognized that epithelial cells participate in innate immune responses, yet the bacterial pattern recognition molecules and signaling pathways are not clear and do not necessarily correspond to those of monocytes, macrophages, and endothelial cells (31). Gingival epithelium is a stratified squamous epithelium surrounding the tooth and forming an attachment to the tooth surface. It functions as a protective barrier against pathogenic microorganisms in dental plaque. The oral mucosa of the gingiva is a useful model for studies of innate immune defenses because it is constantly exposed to microorganisms yet generally maintains a homeostasis and balance that is associated with oral health.

The defensin family of antimicrobial peptides is an evolutionarily conserved group (reviewed in reference 47). In mammals, epithelial defensins include α-defensins of the intestinal epithelium and β-defensins of skin and mucosal epithelia. The β-defensins are small cationic peptides, 36 to 42 amino acids in length (4, 14, 40; J. Harder, J. Bartels, E. Christophers, and J. M. Schröder, Letter, Nature 387:861, 1997), with a structure that is stabilized by three disulfide bonds (reviewed in references 12 and 22). Tracheal antimicrobial peptide (TAP) was the first member of the epithelial β-defensin family characterized (14). Up-regulation of TAP mRNA was shown to occur via the CD14-mediated signal transduction pathway in bovine airway epithelial cells challenged with bacterial lipopolysaccharide (LPS) (13) and with tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) (5, 38). The related lingual antimicrobial peptide (LAP) was shown to be up-regulated in vivo under conditions of infection and inflammation (39).
In humans, two β-defensins, hBD-1 and hBD-2, have been identified exclusively in epithelial tissues. hBD-1 is constitutively expressed in the kidney, pancreas, urinary and respiratory tracts, and oral epithelia (4, 17, 26, 29, 30, 41, 45, 54). hBD-2 was originally isolated from psoriatic-scale keratinocytes (Harder et al., 1997). hBD-2 is poorly expressed in normal epidermal keratinocytes but is induced when keratinocytes are stimulated with gram-negative or gram-positive bacteria, Candida albicans, or TNF-α (Harder et al., 1997), and is up-regulated in inflamed epithelial tissues (27). hBD-2 demonstrates anti-viral activity against yeast and is the first human defensin with gram-negative and gram-positive bacteria (3, 45; Harder et al., 1997).

We previously reported the constitutive expression of hBD-1 mRNA in gingival epithelial cells (26) and the inducible expression of hBD-2 mRNA in cultured gingival epithelial cells (47). In other studies, the expression of hBD-2 mRNA has also been reported to be induced by IL-1β, TNF-α, and specific microorganisms (29; Harder et al., 1997). In this study, we have established that hBD-2 mRNA is expressed in gingival epithelial cells and tissue and that several natural stimuli induce its expression, but expression is regulated differently from that of IL-8, another aspect of innate host defense. We show evidence for the involvement of multiple pathways of regulation and find differences in the abilities of cell walls extracts of two gram-negative and one gram-positive bacteria to elicit the expression of hBD-2 mRNA, present in both healthy and diseased sites (18), and Porphyromonas gingivalis, a periodontal pathogen (19, 42), to induce hBD-2 mRNA. Finally, we have detected hBD-2 peptide in stimulated gingival epithelial cells. Our findings suggest that hBD-2 mRNA is regulated at the transcriptional level via several signaling pathways and that its regulation differs from that of IL-8.
The RNA pellet was resuspended in 20 μl of Tris-EDTA. Five microliters (25%) was used to determine optical density values. The RNA probe (50 ng/μl) was labeled with a nonisotopic labeling kit (BrightStar Psoraleen-Biotin; Ambion). The biotin-labeled RNA probes were stored in 5-μl aliquots at −80°C. The amounts of biotinylated probes used in RPA were about 400 pg for GAPDH and less than 100 pg for hBD-2 and IL-8 for 20 μg of total RNA in each sample.

Methods. Gingival epithelial cells were seeded on coverslips (9 mm) (Belco Glass Inc., Vineland, N.J.) at 2,000/cm² in a 24-well tissue culture plate (Corning Costar Corporation, Cambridge, Mass.) with 1 ml of KGM per well. The cells were grown for 4 to 5 days prior to challenge with F. nucleatum cell wall for 24 h. Cells on the coverslips were washed with phosphate-buffered saline twice, fixed with 4% paraformaldehyde in Sorensen’s buffer for 5 min, and permeabilized with cold acetone on ice for 5 min. The cells were then blocked with 3% normal serum in 0.05% Tween in Tris-buffered saline (TBS) for 20 min and incubated with polyclonal antibody against hBD-2, kindly provided by Tomas Ganz, University of California—Los Angeles, at 1:500 dilution in Tween-20 and with polyconal antibody against IL-8 and TNFα, a housekeeping gene control (Vector Laboratories Inc.) at 1:20 dilution in TBS for 30 min. The cells were rinsed in TBS twice, reacted with DAPI (4',6'-diamidino-2-phenylindole) for 5 min, and rinsed in TBS twice and distilled water once. The coverslips were air dried for 10 min and mounted on slides with mounting medium (Molecular Probes, Eugene, Ore.). Immunofluorescence images were captured by a Photometrics Sensys camera attached to a Nikon microphot-SA epifluorescence microscope. Image capturing was performed with an IP Lab Spectrum program version 3.12. All computer-generated pictures were organized by Adobe Photoshop version 5.0 software using a PowerPC computer.

Immunocytochemistry. Gingival epithelial cells were seeded on coverslips (9 mm) (Belco Glass Inc., Vineland, N.J.) at 2,000/cm² in a 24-well tissue culture plate (Corning Costar Corporation, Cambridge, Mass.) with 1 ml of KGM per well. The cells were grown for 4 to 5 days prior to challenge with F. nucleatum cell wall for 24 h. Cells on the coverslips were washed with phosphate-buffered saline twice, fixed with 4% paraformaldehyde in Sorensen’s buffer for 5 min, and permeabilized with cold acetone on ice for 5 min. The cells were then blocked with 3% normal serum in 0.05% Tween in Tris-buffered saline (TBS) for 20 min and incubated with polyclonal antibody against hBD-2, kindly provided by Tomas Ganz, University of California—Los Angeles, at 1:500 dilution in Tween-20 and with polyconal antibody against IL-8 and TNFα, a housekeeping gene control (Vector Laboratories Inc.) at 1:20 dilution in TBS for 30 min. The cells were rinsed in TBS twice, reacted with DAPI (4',6'-diamidino-2-phenylindole) for 5 min, and rinsed in TBS twice and distilled water once. The coverslips were air dried for 10 min and mounted on slides with mounting medium (Molecular Probes, Eugene, Ore.). Immunofluorescence images were captured by a Photometrics Sensys camera attached to a Nikon microphot-SA epifluorescence microscope. Image capturing was performed with an IP Lab Spectrum program version 3.12. All computer-generated pictures were organized by Adobe Photoshop version 5.0 software using a PowerPC computer.

RESULTS

Expression of hBD-2 mRNA is variable in gingival-tissue samples. To determine expression of hBD-2 in gingival tissue in vivo, total RNA was extracted from clinically normal tissue freshly obtained from 15 individuals undergoing third-molar extraction. RT-PCR was performed for 25 and 28 cycles to semiquantitatively analyze hBD-2 mRNA. HBD-2 mRNA was expressed in 14 of 15 samples tested, and the level of hBD-2 expression varied between subjects (Fig. 1). For comparisons of tissue samples, an epithelium-specific protein, keratin 5, was used to evaluate the relative contribution of the epithelial compartment in each sample. IL-8 mRNA expression was readily detected in a subset of the normal gingival samples (n = 10) (Fig. 1), suggesting tissue activation even though inflammation was not clinically evident in these tissue samples. No correlation between expression of hBD-2 and IL-8 mRNA was found in the samples. For example, there was no hBD-2 expression but there was IL-8 expression in tissue from subject 2, and there was no IL-8 expression but there was hBD-2 expression in tissue from subjects 1, 6, 8, 11, and 12 (Fig. 1).

Stimulated HGE express hBD-2 mRNA and exhibit differential regulation by various stimuli. To examine expression of hBD-2 mRNA in vitro, HGE were isolated from gingival biopsy specimens overlying impacted third molars and cultured for a few passages. The HGE were challenged with cell wall extract of two gram-negative periodontal bacteria, F. nucleatum and P. gingivalis, and two potent activators for epithelial cells, TNFα and PMA, for 24 h. Total RNA was harvested and analyzed by RT-PCR and RPA. HBD-2 mRNA was significantly up-regulated by F. nucleatum cell wall (Fig. 2) and was found in the samples. For example, there was no hBD-2 expression but there was IL-8 expression in tissue from subject 2, and there was no IL-8 expression but there was hBD-2 expression in tissue from subjects 1, 6, 8, 11, and 12 (Fig. 1).
TNF-α (see Fig. 4) in a dose-dependent fashion, as well as by 10 ng of PMA/ml (Fig. 2). The finding of hBD-2 induction by TNF-α is in agreement with a previous study (Harder et al., 1997). In contrast, P. gingivalis cell wall at all doses tested failed to induce hBD-2 mRNA (Fig. 2). Induction of IL-8 and TNF-α mRNAs was also determined as an indication of the state of cell activation. Both cytokines were induced by F. nucleatum cell wall and TNF-α (Fig. 2; see also Fig. 4) but not by P. gingivalis cell wall and PMA (Fig. 2). P. gingivalis cell wall at the maximum dose (100 μg/ml) suppressed the expression of both cytokines (Fig. 2), consistent with a previous study (10). The viability of HGE and the yield of total RNA were checked during each experiment, and no differences were found between untreated and treated cells. The sizes of amplified products were as predicted. The RPO gene is a housekeeping gene used for RT-PCR analysis for hBD-2, IL-8, TNF-α, GAPDH, and RPO. The results shown are representative of three independent experiments. Note that there was no additional effect on hBD-2 induction with the presence of human serum.

Bacterial LPS is a poor stimulant for the induction of hBD-2 mRNA in HGE. E. coli LPS is a known stimulant for bovine tracheal antimicrobial peptide (13). To determine if LPS is the active component in F. nucleatum cell wall extract, purified LPS fraction of F. nucleatum and E. coli LPS were tested for the ability to up-regulate hBD-2 mRNA. HGE were stimulated with various doses of E. coli LPS in comparison with TNF-α (Fig. 4) and various doses of F. nucleatum LPS in comparison with F. nucleatum cell wall (Fig. 5). Total RNA was harvested and analyzed by RT-PCR (Fig. 4 and 5A) and RPA (Fig. 5B). In contrast to significant hBD-2 induction by F. nucleatum cell wall (Fig. 2 and 5A) and TNF-α (Fig. 4), both E. coli and F. nucleatum LPSs poorly induced hBD-2 mRNA (Fig. 4 and 5A). A slight hBD-2 induction was seen in HGE stimulated with the maximum dose (10 ng/ml) of E. coli and F. nucleatum LPSs used in this study (Fig. 4 and 5A). Interestingly, the slight induction observed by RT-PCR (Fig. 5A) was not detected by RPA (Fig. 5B), indicating differences in the sensitivities of the assays. When the mRNA expression for IL-8 was examined, we found doses of 10 ng of E. coli LPS/ml and 10² ng of F. nucleatum LPS/ml or greater induced IL-8 mRNA (Fig. 4 and 5A). 

![Fig. 3. HBD-2 up-regulation assessed by RPA. (A) RPA analysis. Total RNA (20 μg) was probed with three different biotin-labeled RNA probes: IL-8, hBD-2, and GAPDH, a housekeeping gene control. RPA was conducted as described in Materials and Methods. The abbreviations are the same as in Fig. 2. The data shown are representative of three separate experiments. (B) Densitometric analysis of RPA. The relative ratios of hBD-2 and IL-8 to GAPDH were determined as described in Materials and Methods. The y axis represents the ratios; the x axis represents a control sample and samples treated with various doses of different stimulants as shown in panel A. The results are represented as means ± standard deviations of three separate experiments. F.n., F. nucleatum; P.g., P. gingivalis. ](http://iai.asm.org/content/76/7/2908.full)
express MCP-1 under any conditions known to induce hBD-2 stimulants used (Fig. 6). In contrast to HGF, HGE did not (20, 53), were induced in HGF stimulated with all of the in fibroblasts, mononuclear phagocytes, and endothelial cells activation, and for MCP-1, a chemokine known to be expressed well as epidermis. The mRNAs for IL-8, a marker for cell expression of hBD-2 in the epithelial lining of several organs as epithelial tissue, consistent with other reports showing the ex-

Interestingly, the kinetics of IL-8 and TNF-α mRNA expres-

5A). Furthermore, doses of 10^2 ng of F. nucleatum LPS/ml or greater induced TNF-α mRNA (Fig. 5A). Surprisingly, addition of human serum appeared to inhibit up-regulation of IL-8 and TNF-α mRNAs by both bacterial LPSs compared with HGE stimulated with the same dose of LPS in the absence of serum (Fig. 4 and 5A). However, serum did not affect expression of hBD-2 mRNA in any dose of either bacterial LPS (Fig. 4 and 5A) or F. nucleatum cell wall (data not shown).

**HGF do not express hBD-2 mRNA.** HGF were stimulated for 24 h with different stimulants known to up-regulate hBD-2 in HGE. All stimulants failed to induce hBD-2 expression in HGF either in the absence (Fig. 6) or presence (data not shown) of human serum compared with hBD-2 expression induced in HGE by three activators identified in this study, F. nucleatum cell wall (10 μg/ml), PMA (10 ng/ml), and TNF-α (10 ng/ml) (Fig. 6). This indicates that hBD-2 is derived from epithelial tissue, consistent with other reports showing the expression of hBD-2 in the epithelial lining of several organs as well as epidermis. The mRNAs for IL-8, a marker for cell activation, and for MCP-1, a chemokine known to be expressed in fibroblasts, mononuclear phagocytes, and endothelial cells (20, 53), were induced in HGF stimulated with all of the stimulants used (Fig. 6). In contrast to HGF, HGE did not express MCP-1 under any conditions known to induce hBD-2 and IL-8 (Fig. 6). Interestingly, the P. gingivalis cell wall was effective in up-regulating IL-8 and MCP-1 mRNAs in HGE (Fig. 6) but not hBD-2 and IL-8 mRNAs in HGE (Fig. 2 and 3A), indicating the efficacy of this extract.

**Kinetics of hBD-2 mRNA up-regulation.** To study the kinetics of hBD-2 up-regulation in HGE, mRNAs for hBD-2 as well as for IL-8 and TNF-α were analyzed by RT-PCR at different times after HGE were challenged with either 10 μg of F. nucleatum cell wall/ml, 10 ng of PMA/ml, or 80 ng of TNF-α/ml. The results (Fig. 7) show that hBD-2 was up-regulated by at least two different signaling pathways. While hBD-2 induction by F. nucleatum cell wall and TNF-α was rapid (2 to 4 h), that by PMA was not seen until 10 h after stimulation. The induction by these three stimulants continuously increased up to 24 h, the maximum period of incubation in the study (Fig. 7). Interestingly, the kinetics of IL-8 and TNF-α mRNA expression were different from each other and from that of hBD-2.

IL-8 was rapidly up-regulated at 1 h and peaked a short time later with stimulation by all three stimulators (Fig. 7). IL-8 induction by PMA declined to the baseline level at 24 h (Fig. 7). This was consistent with the absence of IL-8 induction in HGE by PMA at 24 h in other experiments (Fig. 2, 3A, and 6). In contrast, TNF-α mRNA was transiently induced during the first few hours with all three stimulators (Fig. 7). The strong and transient induction of TNF-α prior to hBD-2 induction suggests a possible functional role of TNF-α as an intermediary molecule in hBD-2 regulation.

![Image 5](https://example.com/image5.png)

**Fig. 5.** Comparison of hBD-2 induction by F. nucleatum LPS and cell wall. (A) RT-PCR analysis. HGE were stimulated with various doses of F. nucleatum (F.n.) LPS or cell wall in the absence (−) or presence (+) of human serum for 24 h. Total RNA (3 μg) of each sample was used for RT-PCR analysis for hBD-2, IL-8, TNF-α, and RPO. The results shown are representative of three independent experiments. Note that there was slight hBD-2 induction by F. nucleatum LPS at 10^4 ng/ml, but the degree of hBD-2 induction by F. nucleatum LPS was still less than that by 10^4 ng of F. nucleatum cell wall/ml. Note the dose-dependent response for IL-8 and TNF-α by F. nucleatum LPS and an inhibitory effect of serum on up-regulation of these two cytokines. – RT, minus-RT control. (B) RPA analysis of some RNA samples in panel A. Note the dose-dependent induction of hBD-2 by F. nucleatum cell wall; however, the slight increase in hBD-2 expression by F. nucleatum LPS (10^4 ng/ml) observed by RT-PCR was not detected by RPA. The data shown are representative of three independent experiments.

![Image 6](https://example.com/image6.png)

**Fig. 6.** HGF do not express hBD-2 mRNA. Primary HGF were stimulated with different stimulants for 24 h. RT-PCR analysis was performed using total RNA (3 μg) from each sample. Amplification was conducted for 25 cycles for IL-8, MCP-1, and RPO primers and 35 cycles for the hBD-2 primer pair to detect any small amount of expression. Stimulants included 10 μg of F. nucleatum cell wall/ml (F); 10 ng of PMA/ml (P); 10 ng of TNF-α/ml (T); and 1, 10, and 100 μg of P. gingivalis cell wall/ml (P.g.). C, unstimulated HGF. RPO was uniformly expressed. As a positive control, HGE were stimulated with 10 μg of F. nucleatum cell wall/ml (F), 10 ng of PMA/ml (P), and 10 ng of TNF-α/ml (T). The data shown are representative of two independent experiments. – RT, minus-RT control.
Anti-TNF-α does not inhibit hBD-2 induction by *F. nucleatum* cell wall. To determine whether TNF-α functions as an intermediary molecule in hBD-2 regulation, various doses of antibody directed to TNF-α were preincubated with either 10 μg of *F. nucleatum* cell wall/ml or 10 ng of TNF-α (a positive control for inhibition)/ml for 30 min before HGE were challenged for 10 h. Total RNA was harvested and analyzed by RT-PCR (Fig. 8), and IL-8TNF-α antibody (10 μg/ml) completely neutralized the effect of TNF-α on hBD-2 induction compared with no inhibitory effect on hBD-2 induction by IgG1, an isotype antibody control. However, anti-TNF-α had no effect at all on hBD-2 induction by *F. nucleatum* cell wall. Similarly, anti-TNF-α had an inhibitory effect on IL-8 induction by TNF-α but not by *F. nucleatum* cell wall. Anti-TNF-α alone at 10 μg/ml (Fig. 8, lanes A) had no effect on either hBD-2 or IL-8 expression.

hBD-2 induction requires new protein synthesis and new gene transcription. To examine the mechanism(s) for inducible expression of hBD-2 in response to *F. nucleatum* cell wall, HGE were pretreated with either 10 μg of cycloheximide (an inhibitor of protein synthesis) per ml, 1 μg of actinomycin D (an inhibitor of RNA transcription) per ml, or DMSO (vehicle control) 1 h before exposure to *F. nucleatum* cell wall for an additional 6 h. The viability of HGE and total RNA yield were checked after each treatment, and no differences were found between experimental and control untreated cells. Because stimulation was short (only 6 h), the level of hBD-2 mRNA expression was low. Nevertheless, both cycloheximide and actinomycin D completely blocked the up-regulation of hBD-2 mRNA in response to *F. nucleatum* cell wall stimulation, suggesting that both new protein synthesis and new gene transcription are required for *F. nucleatum*-induced hBD-2 mRNA expression (Fig. 9). Pretreatment with vehicle alone showed no difference in hBD-2 mRNA induction. Similar to induction of hBD-2 mRNA, induction of IL-8 mRNA also required new gene transcription because pretreatment with actinomycin D could completely inhibit *F. nucleatum*-induced IL-8 expression (Fig. 9). Interestingly, in contrast to the inhibition of hBD-2 induction by pretreatment with cycloheximide, IL-8 mRNA was induced in the presence of cycloheximide regardless of stimulation with *F. nucleatum* cell wall.

hBD-2 peptide is detected in HGE by immunofluorescence. To investigate expression of hBD-2 peptide in HGE, cells were cultured on coverslips, exposed to *F. nucleatum* cell wall for 24 h, and then reacted with polyclonal antibody against hBD-2. Using immunofluorescence, hBD-2 peptide was detected in the cytoplasm of HGE stimulated with *F. nucleatum* cell wall but not in unstimulated HGE (Fig. 10), consistent with inducible expression of hBD-2 mRNA. The staining revealed a punctate distribution with concentration of the reaction adjacent to the nucleus (Fig. 10). hBD-2 peptide is not detected in every cell, possibly because of variation in staining of individual cells or because hBD-2 peptide is synthesized in a subpopulation of the epithelial cells.

DISCUSSION

Our studies of β-defensin expression in oral gingival epithelia show several new characteristics of the innate immune response. First, hBD-2 expression is seen in clinically noninflamed gingival tissue. Second, inducible expression of hBD-2
some samples were then challenged with 10 samples treated with stimulation (6 h). The arrow indicates a protected fragment of hBD-2 in the sample treated with both CHX and is inhibited only with ACT D. Also note the superinduction of IL-8 mRNA in the induction is inhibited with both CHX and ACT D, while IL-8 mRNA expression hybridization is shown as a normalization control. Note that hBD-2 mRNA panel A was analyzed by RPA as described in Materials and Methods. GAPDH minus-RT control. (B) RPA analysis. Total RNA (20 g) from the samples were compared and showed no difference. Total RNA was extracted and analyzed by RT-PCR. Note that in this experiment, PCR for hBD-2 was performed for 28 cycles, which resulted in an increased background from the samples in panel A was analyzed by RPA as described in Materials and Methods. GAPDH hybridization is shown as a normalization control. Note that hBD-2 mRNA induction is inhibited with both CHX and ACT D, while IL-8 mRNA expression is inhibited only with ACT D. Also note the superinduction of IL-8 mRNA in the sample treated with both CHX and F. nucleatum cell wall and that the degree of hBD-2 expression was lower than that in Fig. 3A due to a shorter time of stimulation (6 h). The arrow indicates a protected fragment of hBD-2 in the samples treated with F. nucleatum and F. nucleatum cell wall plus DMSO.

mRNA occurs in cells via multiple pathways. Third, hBD-2 mRNA is induced by TNF-a and cell wall extract of a representative commensal (F. nucleatum), but not a pathogenic (P. gingivalis) oral microorganism. Fourth, hBD-2 peptide is detected in cultured epithelial cells challenged with F. nucleatum cell wall extract. Taken together, these findings suggest that oral mucosal cells are in an activated state with respect to expression of hBD-2 and that this state is part of the normal barrier function of oral epithelium. We also show that only minimal up-regulation of hBD-2 occurs in response to F. nucleatum or E. coli LPS. Our analyses show consistent differences between hBD-2 and IL-8 regulation (another marker of innate immune response) in response to LPS, in the kinetic analysis, in the requirement for protein synthesis, and in the in vivo tissue analysis. These differences offer evidence that innate immune responses of HGE are differentially regulated and suggest that multiple complex interactions occur between microbial stimulants and host cells.

Gingival epithelium is a useful model for these studies because we can investigate expression of hBD-2 mRNA in gingival tissue as well as its regulation in cultured gingival epithelial cells challenged with natural stimuli found in the oral cavity. We found a dramatic difference in the regulation of hBD-2 by cell wall extracts of F. nucleatum and P. gingivalis. F. nucleatum is a gram-negative anaerobic bacterium which is commonly found in healthy and diseased sites of periodontal tissue (6, 18). Although F. nucleatum is commonly associated with clinical infections of other body sites, this microorganism is not considered causative in periodontal disease. Rather, it is viewed as a “bridge” between early colonizers of the tooth pellicle and the subsequent adherence of pathogenic microorganisms, such as P. gingivalis. F. nucleatum cell wall was found to up-regulate hBD-2 mRNA, as well as IL-8 and TNF-a mRNAs, while P. gingivalis cell wall did not. It has been shown previously that P. gingivalis inhibits the activation of IL-8 by commensal bacteria, including F. nucleatum (10, 28). The absence of hBD-2 mRNA induction by the P. gingivalis cell wall is therefore consistent with the ability of this organism to evade stimulation of host defense mechanisms, while F. nucleatum may help keep gingival epithelial cells in a stimulated state for effective and continuous host defense. Consistent with these in vitro findings, hBD-2 was detected in clinically noninflamed gingival tissue of 14 of 15 subjects in our study, as well as in recent work by Mathews and coworkers (29). Thus, in contrast to the epidermis, in which hBD-2 mRNA is seen primarily in association with inflammation or disease (27; Harder et al., 1997), our results show that clinically noninflamed oral epithelium is in a partially stimulated state and suggest that this may be due to its exposure to oral commensal microorganisms. It will be important to determine if the difference between commensal and pathogenic microorganisms in regulation of β-defensins is seen in other mucosal epithelia that have region-specific ecologies with response to the balance of commensal and pathogenic microorganisms.
Cellular innate immune responses are postulated to be initiated by microbial components via pattern recognition receptors (31). Recent discoveries have shown expression of Toll-like receptors (TLR) on human cells (9, 32, 37) that serve this function. TLR4 is implicated in LPS responses in mice (35, 36, 44). TLR2 mediates the response to bacterial LPS-CD14 signaling in transfected kidney epithelial cell lines (25, 52) and to bacterial lipoprotein (2, 7). It is speculated that there may be one or more TLR expressed in other cell types that mediate recognition of microbial components to signal the human innate immune response. Thus, a logical active bacterial component for hBD-2 regulation in the F. nucleatum cell wall extract is LPS, a major fraction of the crude cell wall extract. In addition, mRNA expression for the bovine β-defensins, TAP and LAP, was previously shown to be mediated via bacterial LPS and a CD14-dependent signaling pathway (13, 38). In contrast to the bovine system, our findings in human gingival epithelial cells show induction of IL-8 mRNA by both E. coli LPS and F. nucleatum LPS and induction of TNF-α mRNA by F. nucleatum LPS but a poor response for hBD-2 mRNA to LPSs of both bacteria compared to the F. nucleatum cell wall. This suggests that another cell wall component(s) stimulates hBD-2 mRNA induction. Up-regulation of hBD-2 mRNA in gingival epithelial cells in response to E. coli LPS was previously shown by Mathews and coworkers (29); however, the stimulation in their study was also low compared to the effect of the proinflammatory cytokine IL-1β. Addition of serum as a source of soluble CD14 and LPS-binding protein (LBP) (24) does not activate or inhibit hBD-2 induction in gingival epithelial cells stimulated by bacterial LPS. The addition of serum inhibited induction of IL-8 and TNF-α, possibly because serum proteins, such as LBP and others, enable binding and neutralization of LPS (48, 49, 50). Finally, hBD-2 induction by F. nucleatum cell wall was not inhibited by pretreatment of the cell wall extract with polymyxin B sulfate, an inhibitor of LPS signaling (data not shown). These findings indicate that, unlike induction of cytokines, hBD-2 up-regulation in gingival epithelium is poorly responsive to bacterial LPS; they also support a CD14-independent pathway and suggest multiple cellular responses for eliciting various aspects of innate immune defenses in epithelial cells. Our results are in agreement with findings for human uroepithelial cells that respond poorly to E. coli LPS but effectively to P-fimbriated E. coli cell wall extracts (21) and with other examples of human epithelial cell signaling of innate immune responses that differ from those of monocytes, macrophages, etc. (15, 24).

Kinetic analysis of hBD-2 mRNA induction indicates involvement of multiple signaling pathways and further supports the distinction between hBD-2 and IL-8 regulation. HBD-2 mRNA is rapidly induced by F. nucleatum cell wall and TNF-α and accumulates during the 24-h period of study, as would be expected for an innate immune defense response to microbial and inflammatory stimuli. In contrast to the profile of hBD-2 induction, induction of TNF-α is rapid and transient, while that of IL-8 is rapid but with longer duration. We thought that the transient induction of TNF-α might indicate its role as an intermediary molecule involved in hBD-2 up-regulation by all three stimulators. This hypothesis was disproved (Fig. 8) by using a specific antibody to neutralize both exogenously added and endogenously synthesized TNF-α.

The accumulation of hBD-2 mRNA may be due to new gene transcription, altered mRNA stability, or both, since inhibitors of both transcription and translation block hBD-2 mRNA expression. The inhibitory effect of actinomycin D on hBD-2 and IL-8 mRNA induction implies that both genes are regulated at the transcriptional level. The inhibition of hBD-2 induction by cycloheximide implicates the requirement for new protein synthesis for hBD-2 induction by F. nucleatum cell wall; these proteins may include cell receptors, intermediary proteins in signaling pathways, transcription factors, or proteins that alter mRNA stability. Elevated levels of IL-8 mRNA following inhibition of protein synthesis can be most readily explained by loss of rapidly turned-over proteins necessary for RNA degradation. This is very likely the case for cytokine genes such as IL-8, whose transcripts contain an AU-rich element, a specific binding site for labile proteins, in the 3′ untranslated region (1, 46).

Immunofluorescence detection of hBD-2 peptide within the cytoplasm of stimulated gingival epithelial cells is consistent with the inducible expression of hBD-2 mRNA. The punctate perinuclear immunostaining is suggestive of endoplasmic reticulum and Golgi apparatus localization, typical of a secreted product with a signal peptide, such as is found in both hBD-1 and hBD-2 mRNAs (17, 41, 45; Harder et al., 1997).

In conclusion, our results indicate that gingival epithelial cells and tissue express messages for hBD-2 and produce hBD-2 peptide in response to inflammatory mediators and to continuous challenges from the cell walls of commensal bacteria which are naturally present in the oral cavity. Interestingly, the cell wall of a periopathogenic bacterium, P. gingivalis, does not induce hBD-2 mRNA and therefore appears to have a strategy of evading this aspect of the host innate immune response, in addition to others shown previously (10, 28). The difference in stimulation by commensal and pathogenic bacteria may be important in understanding the molecular aspects of host-bacterial interaction as well as for potential new preventive therapies for mucosal infection. The production of hBD-2 as part of the epithelial barrier may be especially important in innate host defense at confrontational mucosal sites in the gingival sulcus and therefore may contribute to overall oral health and disease susceptibility.

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