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Antimicrobial Barrier of an *in vitro* Oral Epithelial Model

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Objective

Oral epithelia function as a microbial barrier and are actively involved in recognizing and responding to bacteria. Our goal was to examine a tissue engineered model of buccal epithelium for its response to oral bacteria and proinflammatory cytokines and compare the tissue responses with those of a submerged monolayer cell culture.

Design—The tissue model was characterized for keratin and β -defensin expression. Altered expression of β -defensins was evaluated by RT-PCR after exposure of the apical surface to oral bacteria and after exposure to TNF- α in the medium. These were compared to the response in traditional submerged oral epithelial cell culture.

Results—The buccal model showed expression of differentiation specific keratin 13, hBD1 and hBD3 in the upper half of the tissue; hBD2 was not detected. hBD1 mRNA was constitutively expressed, while hBD2 mRNA increased 2-fold after exposure of the apical surface to three oral bacteria tested and hBD3 mRNA increased in response to the non-pathogenic bacteria tested. In contrast, hBD2 mRNA increased 3–600 fold in response to bacteria in submerged cell culture. hBD2 mRNA increased over 100 fold in response to TNF- α in the tissue model and 50 fold in submerged cell culture. Thus, the tissue model is capable of upregulating hBD2, however, the minimal response to bacteria suggests that the tissue has an effective antimicrobial barrier due to its morphology, differentiation, and defensin expression.

Conclusions—The oral mucosal model is differentiated, expresses hBD1 and hBD3, and has an intact surface with a functional antimicrobial barrier.

Keywords

buccal mucosa; β -defensin; Gram-negative bacteria; commensal bacteria

Introduction

Oral epithelia play a critical role in their function as a microbial barrier. This role is necessitated by the presence of the large number and variety of microbes that are present in the oral cavity that may adhere to the epithelia or tooth surfaces. It has been estimated that over 500 species of bacteria can be found in the oral cavity (Kolenbrander, 2000). These bacteria can be categorized as non-pathogenic (commensal) or pathogenic. Pathogenic bacteria include Gram positive *Streptococcus mutans* associated with dental caries, and the Gram negative *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, and others associated with periodontal disease (Haffajee and Socransky, 1994; Ximenez-Fyvie et al., 2000). The epithelium is now recognized to be actively involved in the recognition and response to bacteria acting in concert with neutrophils in the innate immune response to infection (Dale, 2002;

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Darveau et al., 1998; Tonetti et al., 1998). The functional epithelial barrier against microbes has two main parts; first, the physical barrier consisting of closely adherent cells, cell-cell junctions, and a complex and continuous differentiation pattern, and second, the epithelial innate immune response that includes the expression of beta-defensins and other antimicrobial peptides and the expression of cytokines such as IL-8, as a chemoattractant for neutrophils.

The human beta-defensins (hBD) are antimicrobial peptides that are expressed by epithelia throughout the body including epithelia of the oral cavity. There are now 28 known beta-defensin genes (Schutte et al., 2002), however, expression of hBD1, 2, and 3 has been most investigated. The peptides are produced by oral epithelial cells and may act to control the many commensal and pathogenic bacteria in the oral cavity (Weinberg et al., 1998). Regulation of these defensins is modulated by differing mechanisms. hBD1 is generally considered to be constitutively expressed (Krisanaprakornkit et al., 1998; Mathews et al., 1999), and may be upregulated by bacterial products in some epithelia (Chronnell et al., 2001; Sorensen et al., 2005). hBD2 is strongly upregulated *in vitro* by both commensal and pathogenic bacteria as well as by proinflammatory cytokines. hBD2 expression is also amplified by interaction with monocytes via IL1 β (Harder et al., 1997; Liu et al., 2003). Finally, hBD3 is expressed in normal epithelium and is upregulated by bacteria, IFN γ and growth factors (Harder et al., 2001; Sorensen et al., 2005).

β -Defensin expression and the epithelial differentiation pattern are likely to work together to provide a barrier to microbes. Expression of the inducible hBD2 in normal oral tissues *in situ* is thought to be due to the constant exposure of the tissue to bacteria within the oral cavity, and is an indication of the enhanced state of immune readiness of oral epithelia (Dale, 2002). Both the constitutive hBD1 and the inducible hBD2 are expressed in the suprabasal layers of normal gingival epithelia (Krisanaprakornkit et al., 2000) and in buccal epithelium (Sun et al., 2005). However, buccal epithelium displays a non-keratinized pattern of differentiation and is more permeable to various test substances (Wertz and Squier, 1991) and may be more permeable to microbial products.

The goal of this project was to examine a buccal tissue model for its response to oral bacteria emphasizing the expression of antimicrobial peptides of the β -defensin family. We compared this *in vitro* tissue model and those of a submerged monolayer culture model for responses to commensal and pathogenic bacteria and proinflammatory cytokines to test the hypothesis that the three dimensional structure of the tissue model results in altered response to bacterial challenge that may be more representative of the tissue *in vivo*.

METHODS

Oral Tissue and Cell Culture Preparation and Use

The tissue model described here is an organotypic culture of normal human oral keratinocytes cultured in serum free medium to form a three-dimensional differentiated tissue, which histologically is similar to buccal mucosa (ORL-100, EpiOralTM, MatTek Corporation, Ashland, MA). Cell culture inserts are coated with an extracellular matrix preparation upon which gingival keratinocytes are seeded. After several days of submerged culture, the culture inserts containing the developing tissues are elevated to the air liquid interface, which induces stratification and differentiation as described (Klausner et al., in preparation¹). Growth medium is placed beneath the culture inserts and thus the tissue is nourished by medium which permeates through the microporous membrane on the bottom of the cell culture inserts. Differentiating cultures were obtained in groups of 24 units. Upon receipt individual cultures

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were placed at the air liquid interface in 6-well plates with 5 ml of serum free minimal media containing growth factors (MatTek proprietary media) and rested overnight (37°C, 5% CO₂). Stimulation of cultures was then initiated (T=0).

The oral epithelial monolayer culture model was used as previously described (Krisanaprakornkit et al., 1998). Gingival epithelial cells were derived from tissue obtained from extraction of third molars according to procedures approved by the University of Washington IRB. Cultures were grown to 80% confluence in serum free keratinocyte basal medium (KBM) containing bronchial epithelial growth medium (BEGM) supplements omitting retinoic acid (Cambrex Corp) with 0.03mM Ca⁺⁺, then shifted to 0.15mM Ca⁺⁺ medium 24 h before stimulation to facilitate differentiation as necessary for β-defensin expression.

Bacterial growth and tissue/cell challenge

Porphyromonas gingivalis (ATCC 33277 or strain 861) bacterial cells were cultured in anaerobic conditions (85% N₂, 10% H₂, 5% CO₂) at 37°C in Trypticase soy broth (BBL, Sparks, MD) supplemented with 1 g of yeast extract, 5 mg of hemin and 1 mg of menadione per liter. *Streptococcus gordonii* DL-1 was grown in Trypticase soy broth at 37°C under static conditions. *Fusobacterium nucleatum* ATCC 25586 was grown in Todd-Hewitt broth supplemented with 1 g of yeast extract per 100 ml at 37°C in anaerobic conditions. Bacterial numbers were estimated by density in a GENios Multi-detection Reader (TECAN US, Research Triangle Park, NC).

Bacterial challenge to the tissues typically consisted of 6×10⁶ bacteria in 10–50 μl bacterial growth medium which was placed on the apical tissue surface. This dose of bacteria was estimated to be equivalent to a ratio of 100:1 bacteria per surface cell. Bacteria were pelleted and resuspended in fresh growth medium. The bacterial mixture or medium (control) was placed on the surface of the tissue. Bacteria were added to the submerged gingival epithelial cells at a ratio of 100:1. Stimulation with either TNFα or IL-1β (100 ng/ml; Cell Sciences, Inc., Norwood MA) was via culture medium for both cell culture and the tissue model.

Histological analysis

Tissue model samples were fixed in 10% formalin or methyl Carnoy's fixative and cut from the cell culture inserts using an 8-mm diameter dermal punch and cuticle scissors. Fixed tissues were embedded in paraffin and 5–7 micron thick cross sections were cut. The sectioned tissues were then processed using hematoxylin and eosin (H&E) or prepared for immunohistochemistry.

Procedures for immunohistochemistry followed those of Dale *et al.* (Dale et al., 2001) for routine histology. Sections were deparaffinized and rehydrated; endogenous peroxide was blocked using 1% H₂O₂/Tris buffered saline (TBS). After blocking with 3% normal serum of the source of secondary biotinylated antibody, the sections were incubated with primary antibody. A monoclonal antibody to cytokeratin K13, K14, and polyclonal antibodies to beta-defensins hBD1, hBD2, and hBD3 were used as described (Dale et al., 2001). Detection was via the avidin-biotin-peroxidase complex method using diaminobenzidine as substrate (Vector Laboratories, Burlingame CA). Rabbit polyclonal antibodies to hBD1 and hBD2 were a generous gift from Dr. T. Ganz, UCLA; rabbit polyclonal antibody to hBD3 was from Orbigen, Inc. (San Diego, CA); and mouse monoclonal antibody LL001 to human keratin 14 and mouse monoclonal AE8 to human keratin 13 were from Abcam (Cambridge, MA).

Tissue and Cell analysis

RT-PCR—Total RNA was extracted from keratinocytes or tissues using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's suggestion. cDNA was prepared using 1 µg total RNA with the RETROscript kit (Ambion, Inc., Austin TX). Controls without RT enzyme were included in each experiment. Amplification of the resulting cDNA was carried out with each 50 µl of PCR mixture containing 3 µl cDNA, 1X PCR buffer, 1.5 mM MgCl₂, 10 mM dNTP mix, 250 nM each of sense and antisense primers, and 2.5 U of Taq DNA polymerase. Ribosomal phosphoprotein (RPO) and GAPDH were used as housekeeping control genes to determine the total RNA level. PCR conditions and primer sequences are summarized in Table 1. The primers for hBD2 and RPO have been previously described (Harder et al., 2001; Krisanaprakornkit et al., 1998).

Quantitative Real-time PCR—cDNA was analyzed using the iCycler system (Bio-Rad, Hercules, CA) for quantitative real-time PCR using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). The reaction was set up in a 96-well plate, each well containing 12.5 µl of SYBR Green mix, 2 µl of cDNA, and 2 µM primers. The amplification conditions were initial denaturation at 95°C for 12 min followed by 40 cycles of denaturation at 95°C for 30s, annealing at 57–65°C for 30s, and elongation at 72°C for 60s. Melt-curve analysis was performed to confirm that the detected signal was that of SYBR Green binding to the expected amplification product and not to the possible primer-dimers. Oligonucleotide primers were designed according to the published sequences (Table 1). In initial experiments, amplification efficiency was determined for all primer pairs. Amplification was performed in duplicate and normalized to housekeeping genes RPO or GAPDH. Results are expressed as the relative fold increase of the stimulated samples over the controls, referred to as Pfaffl's method (Pfaffl, 2001).

RESULTS

Morphology and Differentiation

A comparison of buccal epithelium and the oral tissue model is shown (Fig 1A, B). The oral tissue model is stratified with 15–30 cell layers (Fig. 1B) and has a typical non-keratinized morphology with basal, intermediate, and superficial layers in which the cells retain their nuclei. Neither the tissue nor the model contains a stratum corneum or granular layer. The oral tissue model has a flat interface with the supporting membrane in contrast to buccal tissue, which has a sinuous interface with the underlying lamina propria. Keratins were examined as markers of differentiation within the tissue model. Keratin 14 (Fig. 1B), a basal cell marker, is expressed most strongly in the lower half of the tissue. Keratin 13 (Fig. 1C), a differentiation marker for non-keratinized oral tissue (Presland and Dale, 2000), is expressed in the upper half of the tissue. *In vivo*, K14 is typically limited to the basal cell layer and 1–2 suprabasal layers, and K13 is found in all layers above the basal layer. Thus, the overall differentiation pattern is similar, but not identical, to the *in vivo* pattern.

Expression and localization of the human beta defensins was also examined (Fig. 1 D-F). In the oral tissue model, the constitutive hBD1 was expressed suprabasally and the inducible hBD2 was not detected. hBD3 was also detected in the suprabasal layers. This peptide is typically present in oral tissues (Dunsche et al., 2002), and is also inducible.

Response to oral bacteria and proinflammatory cytokine challenge

Oral commensal (*S. gordonii* and *F. nucleatum*) and pathogenic (*P. gingivalis*) bacteria were incubated with the buccal tissue model for 24–72 hr and the epithelial response evaluated by the expression of mRNA for β -defensin-1, -2, and -3 by RT-PCR (Fig. 2). hBD1 mRNA was constitutively expressed and showed little or no variation between epithelium incubated with

bacteria vs. the control. hBD1 mRNA also showed little variation with time of incubation from time 0 to 48 hr. hBD3 mRNA was also expressed both with and without stimulation. In those experiments in which hBD3 was upregulated, it was usually upregulated by commensal bacteria (Fig. 2A) but this was variable between experiments. On the other hand, hBD2 mRNA expression was consistently upregulated in response to each of the bacteria tested. HBD2 was also upregulated by the bacteria-free conditioned medium from the *P. gingivalis* culture that contains secreted proteases (Curtis et al., 2001). Quantitative PCR showed approximately 2-fold upregulation of hBD2 in the tissue for each bacteria relative to its media control (Fig. 2B). Upregulation of hBD2 was not affected by the presence or absence of antibiotics in the growth medium, and was not enhanced when bacterial exposure was done in the presence of 0.1% human serum as a source of soluble CD14 and lipopolysaccharide binding protein (LBP), which serve as cofactors in the response of TLR4 to bacterial LPS (data not shown). Several other markers of innate immunity were also evaluated. IL-8, TLR2, TLR4, PAR-1 and PAR-2 were all detected in the oral tissue model by RT-PCR. Only IL-8 and TLR2 mRNAs showed possible upregulation of expression in the presence of bacteria (Fig. 2A and 3).

The hBD2 response of monolayer oral epithelial cell cultures to oral bacteria was compared with that of the tissue model. Expression of hBD2 was evaluated by quantitative PCR in three different donor cell lines and ranged from 17–600 fold in response to *P. gingivalis*, 3–5 fold in response to *S. gordonii*, and 12–28 fold in response to *F. nucleatum* (Table 2). The results suggest that the tissue model is much less responsive than the cell cultures to bacteria.

Because the hBD2 response to bacteria in the tissue model was low, we asked if the cells of this model were capable of significant levels of upregulation of hBD2 mRNA. The tissue model was stimulated with TNF- α and IL1 β and results analyzed by quantitative PCR. HBD-2 mRNA was upregulated approx. 15-fold at 48 hr by IL1 β (100 ng/ml), and 55–200-fold by TNF- α at 24 hr (100 ng/ml). HBD2 in monolayer cultures was upregulated approximately 50 fold by TNF- α (triplicate analysis in one donor cell line) (Table 2). Verification of hBD2 peptide expression in the TNF- α stimulated tissue model is shown in Figure 4. HBD2 expression is upregulated in cells within the lower half of the epithelial tissue model when TNF- α was placed in the medium below the tissue.

DISCUSSION

Defensin expression has been previously shown in multiple regions of oral mucosa, in epidermal or oral epithelial cell culture as well as in organotypic epidermal tissue and skin substitute models (Chadebech et al., 2003; Krisanaprakornkit et al., 2000; Liu et al., 2002; Liu et al., 2003; Lu et al., 2005; Supp et al., 2004). In the present report, we extend these observations to an oral epithelial tissue model which most closely resembles buccal epithelium. Both hBD1 and hBD3 peptides are expressed constitutively in this model in the suprabasal cell layers, while the inducible hBD2 is poorly expressed. We show that this oral epithelial model responds to bacterial challenge when the bacteria were presented on the tissue apical surface. Because of the known poor response of oral epithelial cells to purified LPS (Krisanaprakornkit et al., 2000; Liu et al., 2003), we used bacterial cultures of commensal and pathogenic organisms, which elicit upregulation of hBD2 mRNA in cell culture by several signaling pathways (Chung and Dale, 2004; Chung et al., 2004; Krisanaprakornkit et al., 2002). Surprisingly, there was only minimal response by the tissue model while the same bacteria resulted in 3–600 fold upregulation of hBD2 mRNA in submerged monolayer cell cultures. Nevertheless, the tissue model is capable of expressing hBD2 mRNA and showed a 50–200 fold upregulation of hBD2 in response to TNF- α in the culture medium, similar to the response in the monolayer cultures. The dramatic difference in upregulation appears to be due to the fact that TNF- α can diffuse through the supporting membrane (pore size = 0.4 μ m) into the tissue model, while the bacteria were presented on the tissue apical surface. Thus, the weak

response to bacteria may be due to poor penetration of the bacterial signal into the epithelium, suggesting that the tissue offers an effective antimicrobial barrier. This type of barrier would be anticipated in a keratinized gingival model, but also appears operative in the buccal tissue model with a non-keratinized differentiation pattern. An effective barrier to permeability by water was shown in some other buccal epithelial tissue models (Selvaratnam et al., 2001), and our study, by implication, extends these findings to an antimicrobial barrier.

In gingival and buccal tissue, both hBD1 and hBD2 peptides are expressed in the suprabasal layers (Dale et al., 2001; Sun et al., 2005). In contrast, hBD2 is only expressed following bacterial challenge in most regions of skin (Ali et al., 2001) or in inflammatory disorders like psoriasis (Harder et al., 1997). Expression of hBD2 in normal oral tissues in situ is thought to be due to constant exposure of the tissue to bacteria within the oral cavity (Dale and Fredericks, 2005), obviously not the case in the tissue culture model grown under sterile conditions. hBD3 expression has been demonstrated in numerous oral sites (Dunsche et al., 2002) and localized in the basal cell layer in gingival tissue (Lu et al., 2005) and the upper layers of epidermis (Sayama et al., 2005). The constitutive hBD3 expression seen here localized in the suprabasal layers could result from the presence of EGF in the growth medium. EGF is a known regulator of hBD3 expression (Sorensen et al., 2005). Thus, hBD3 may contribute to the antimicrobial barrier of the tissue model, especially since hBD3 has been shown to have potent antimicrobial and antifungal activity (Harder et al., 2001; Joly et al., 2004).

Purely epithelial tissue models have a limited response to purified bacterial products, such as LPS, for upregulation of hBD2 mRNA or peptide (Chadebech et al., 2003), but the response is strongly enhanced in the presence of mononuclear leukocytes and IL1 β (Liu et al., 2003; Sorensen et al., 2005). Oral epithelial cells in monolayer culture also respond poorly to bacterial LPS (Krisanaprakornkit et al., 2000). In contrast, oral and skin epithelial cells strongly upregulate hBD2 mRNA in response to whole bacteria or bacterial cell wall preparations apparently due to their response to multiple bacterial components (Chung and Dale, 2004; Krisanaprakornkit et al., 2000) including proteases of *P. gingivalis* (Chung et al., 2004). In the present study, *P. gingivalis* culture supernatant, presumably containing gingipains, also upregulated hBD2 mRNA in the tissue model, consistent with previous observations in cell culture in which the role of PARs was implicated in hBD2 regulation (Chung et al., 2004) and consistent with the expression of both PAR1 and PAR2 in the tissue model (Fig. 3).

The extent of upregulation of hBD2 in the tissue model was very limited in contrast to results in monolayer cell culture, even with stimulation by whole bacteria. In situ, other cell types (lymphocytes and dendritic cells) in the tissue produce proinflammatory cytokines that contribute to the epithelial cell hBD2 response. But the lack of other cell types in the model does not explain the difference in hBD2 response of the tissue model and cell culture since only epithelial cells are present in both conditions. Thus, the contrast between the epithelial tissue model and the monolayer culture model seems to be a result of an epithelial antimicrobial barrier associated with stratification, differentiation, and constitutive expression of hBD1 and hBD3. Nevertheless, the tissue model is responsive and can produce high levels of hBD2 peptide as confirmed by the response to TNF- α and IL-1 β when added directly to the medium.

In the oral cavity the buccal epithelium is regularly perturbed by mechanical forces in mastication, acids in food or those produced by bacteria, proteases in saliva, toothpaste, alcohol, thermal insult, etc. and responds effectively to bacteria by producing antimicrobial peptides including hBD2. The oral mucosal model is differentiated, expresses hBD1 and hBD3, and has an intact sterile surface with a functional antimicrobial barrier. This model can serve as a useful basic tool for the study of tissue innate immune responses as purely epithelial model or alternatively can be modified by incorporation of additional cell types.

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Figure 1. Histology and immunohistochemistry of oral tissue model

A. Buccal tissue, H&E stained section.

B. Oral tissue model, H&E stained section.

C. – G. Oral tissue model immunohistochemical staining with antibodies to Keratin 14 (C); Keratin 13 (D); hBD1 (E); hBD2 (F); hBD3 (G). Note that the differentiation specific keratin 13 is expressed in the upper portion of the tissue. hBD1 and hBD3 are also expressed in the upper layers, while hBD2 is not detected. Original magnification 10X.

Figure 2. Expression of β -defensins and IL-8 in the oral tissue model in response to commensal and pathogen oral bacteria

The tissue model was exposed for 24 h to *P. gingivalis*, *S. gordonii*, or *F. nucleatum* (as indicated by +) or to growth medium (-) placed on the tissue surface as described in Methods section. Total RNA was extracted and analyzed for various markers of innate immunity.

A. RT-PCR analysis for hBD1–3, IL8 and RPO in two independent experiments. Note that hBD1 is constitutively expressed, while hBD2 is upregulated with all bacteria and hBD3 is upregulated with the commensals (*S. gordonii* and *F. nucleatum*).

B. Quantitative PCR for change in hBD2 mRNA expression in the oral tissue model in response to bacteria at 24 h exposure. Expression of hBD2 is shown relative to GAPDH and compared to the growth medium for each bacterial species (results are the average of 3–5 independent experiments).

Figure 3.

Expression of the receptors TLR2, TLR4, PAR1, and PAR2 mRNA in the oral tissue model in response to commensal and pathogen oral bacteria as in Figure 2. T=0, control tissue at the time of initial challenge; T=+24, control tissue with no challenge.

Figure 4. hBD2 immunostaining in TNF- α stimulated (A) and control (B) buccal tissue model. Note hBD2 staining in distinct cells within the lower half of the epithelial tissue model in response to TNF- α placed in the medium under the tissue. Exposure to TNF- α was for 48 hr. Original magnification 10X

Table 1

PCR Primers and conditions

Product	Forward Primer (5'–3')	Reverse primer (5'–3')	T _m (°C)	Prod (bp)
<i>Primers for RT-PCR:</i>				
hBD1	CGC CAT GAG AAC TTC CTA CC	CAC TTG GCC TTC CCT CTG TA	52	207
hBD2	CCA GCC ATC AGC CAT GAG GGT	GGA GCC CTT TCT GAA TCC GCA	65	255
hBD3	AGC CTA GCA GCT ATG AGG ATC	CTT CGG CAG CAT TTT GCG CCA	62	206
TLR2	GGC CAG CAA ATT ACC TGT GTG	CCA GGT AGG TCT TGG TGT TCA	65	612
TLR4	CCA GGT AGG TCT TGG TGT TCA	TCC CAC TCC AGG TAA GG=TG TT	55	623
PAR1	TGT GAA CTG ATC ATG TTT ATG	TTC GTA AGA TAA GAG ATA TGT	60	708
PAR2	GCA GCC TCT CTC TCC TGC AGT GG	CTT GCA TCT GCT TTA CAG TGC G	60	1066
IL-8	TTT CTG ATG GAA GAG AGC TCT GTC TGG	AGT GGA ACA AGG ACT TGT GGA TCC TGG	60	598
RPO	AGC AGG TGT TCG ACA ATG GCA	ACT CTT CCT TGG CTT CAA CCT	60	342
GAPDH	CCA CCC ATG GCA AAT TCC ATG GCA	TCT AGA CGG CAG GTC AGG TCC ACC	62	500
<i>Primers for real-time PCR:</i>				
hBD2	As above			
GAPDH	CAA AGT TGT CAT GGA TGA CC	CAA TGG AGA AGG CTG GGG	50	195
RPO	CCA GCC ATC AGC CAT GAG GGT	GCC TTG ACC TTT TCA GCA AG	59	213

Table 2Comparison of hBD2 Upregulation in Tissue Model and Cell Culture in Response to Oral Bacteria and TNF- α

Stimulant ¹	Time	Mean hBD2 fold increase	
		Tissue model (n) ²	Cell culture (n) ³
<i>S. gordonii</i> ⁴	24h	2.25 (4) range 1.8–2.5	4 (2) range 3–5
<i>F. nucleatum</i>	24h	2.28 (5) range 1.2–2.7	25 (2) range 12–38
<i>P. gingivalis</i> ⁵	24h	1.83 (3) range 1.7–2.0	259 (3) range 17–600
TNF- α (100 ng/ml)	24h	128 (2) range 55–202	50 (3) range 41–65
IL-1 β (100 ng/ml)	48h	15 (1)	N.T.

¹ Bacterial stimulants were placed on the apical tissue surface, or in culture medium for cell culture. Cytokines were placed in the medium beneath the tissue surface, or in cell culture medium.

² For bacterial stimulation, fold increase by quantitative PCR relative to bacterial media control; values normalized to GADPH. For TNF- α and IL-1 β , fold increase relative to T=0; values normalized to GADPH.

³ Fold increase by quantitative PCR relative to unstimulated control; values normalized to RPO.

⁴ Bacterial samples were pelleted and resuspended in fresh medium and used as described in Methods.

⁵ Two similar strains of *P. gingivalis* were used (ATCC 33277 and strain 381).

(n) number of independent experiments; N.T., not tested.