Activation of protective responses in oral epithelial cells by *Fusobacterium nucleatum* and human β -defensin-2

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Oral epithelia are constantly exposed to non-pathogenic (commensal) bacteria, but generally remain healthy and uninfamed. *Fusobacterium nucleatum*, an oral commensal bacterium, strongly induces human β -defensin-2 (hBD2), an antimicrobial and immunomodulatory peptide, in gingival epithelial cells (GECs). hBD2 is also expressed in normal oral tissue leading to the hypothesis that oral epithelia are in an activated state with respect to innate immune responses under normal *in vivo* conditions. In order to test this hypothesis, global gene expression was evaluated in GECs in response to stimulation by an *F. nucleatum* cell wall (FnCW) preparation and to hBD2 peptide. FnCW treatment altered 829 genes, while hBD2 altered 209 genes ($P < 0.005$, ANOVA). Many induced genes were associated with the gene ontology categories of immune responses and defence responses. Consistent with the hypothesis, similar responses were activated by commensal bacteria and hBD2. These responses included up-regulation of common antimicrobial effectors and chemokines, and down-regulation of proliferation markers. In addition, FnCW up-regulated multiple protease inhibitors, and suppressed NF- κ B function and the ubiquitin/proteasome system. These global changes may protect the tissue from inflammatory damage. Both FnCW and hBD2 also up-regulated genes that may enhance the epithelial barrier. The findings suggest that both commensal bacteria and hBD2 activate protective responses of GECs and play an important role in immune modulation in the oral cavity.

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INTRODUCTION

Epithelia of the body surface and mucosa are in continuous contact with heterogeneous populations of diverse commensal micro-organisms, apparently without detriment to the host. The stratified epithelia of the oral cavity and skin not only form a physical barrier, but there is increasing recognition that epithelial cells also actively participate in innate immunity and provide an antimicrobial barrier (Ganz, 2003). In addition, commensal bacteria influence the development of mucosal innate immune responses to pathogenic bacteria (Collier-Hyams & Neish, 2005; Kelly *et al.*, 2005; Macpherson & Harris, 2004; Sansonetti, 2004). The human oral cavity contains approximately 10^{10} bacteria, representing more than 500 species, inhabiting the surface of the teeth, gingival crevices, buccal

mucosa and tongue; many of these bacteria are commensals (Socransky *et al.*, 1998). Thus, oral epithelial cells are especially suitable for examining cellular responses to commensal bacteria. One beneficial effect of commensal bacteria is the induction of human β -defensins (hBDs) (Krisanaprakornkit *et al.*, 2000; Wehkamp *et al.*, 2004). These peptides have antimicrobial activity, and also immunomodulatory functions that link innate and adaptive immunity (Boniotto *et al.*, 2006; Niyonsaba *et al.*, 2004; Yang *et al.*, 1999, 2004).

Fusobacterium nucleatum belongs to the family *Bacteroidaceae* and is a dominant micro-organism within the periodontium in the healthy oral cavity (Kolenbrander *et al.*, 2002). *F. nucleatum* is not responsible for destructive periodontal disease, which is a major cause of tooth loss. It is an intermediate colonizer bridging attachment of commensals that colonize the tooth and epithelial surface with true pathogens (Kolenbrander, 2000; Kolenbrander *et al.*, 2002). *F. nucleatum* and *F. nucleatum* cell wall (FnCW) extracts induce expression of hBD2 in cultured primary human gingival epithelial cells (GECs) *in vitro* (Krisanaprakornkit *et al.*, 2000). In most epithelia *in vivo*, hBD2 is found only in

Abbreviations: EU, endotoxin unit; FnCW, *Fusobacterium nucleatum* cell wall; GEC, gingival epithelial cell; GO, gene ontology; hBD, human β -defensin; IL, interleukin; PgCW, *Porphyromonas gingivalis* cell wall; QRT-PCR, quantitative real-time PCR; TNF, tumour necrosis factor.

A table of GEC gene expression data is available as supplementary material with the online version of this paper.

inflamed tissue (Liu *et al.*, 1998), but both healthy and inflamed gingival tissue express hBD2 (Dale *et al.*, 2001; Krisanaprakornkit *et al.*, 2000; Lu *et al.*, 2004). Interestingly, induction of hBD2 in GECs in response to FnCW is mainly through MAPK signalling, and not NF- κ B signalling, consistent with its expression in uninfamed oral tissue (Chung & Dale, 2004; Krisanaprakornkit *et al.*, 2002). These observations led to the hypothesis that innate immune responses of normal uninfamed oral epithelia, exemplified by hBD2, are primed for response to subsequent exposure to oral pathogens. This may be due to the presence of commensal bacteria or to hBD2 itself, which has been shown to act in a cytokine-like manner toward dendritic cells, peripheral blood mononuclear cells and lung epithelial cells (Boniotto *et al.*, 2006; Durr & Peschel, 2002; Niyonsaba *et al.*, 2005; Yang *et al.*, 1999). To test this hypothesis, we evaluated the global gene expression of GECs under conditions that mimic the *in vivo* condition. Thus we tested GEC gene expression in response to the commensal FnCW preparation and to hBD2 peptide using oligonucleotide-based microarrays (Affymetrix). We show that both FnCW and hBD2 induce significant changes in the expression of genes associated with immune and defence responses. These findings support the hypothesis that commensal bacteria modulate gene expression resulting in the enhanced immune readiness of epithelial cells, and that hBD2 acts as a cytokine with effects on epithelial cells.

METHODS

Chemicals and reagents. Bacterial crude cell wall extracts from *F. nucleatum*, subspecies *nucleatum* (ATCC 25586) and *Porphyromonas gingivalis* (ATCC 33277) were prepared as previously described (Krisanaprakornkit *et al.*, 1998, 2000). hBD2 was purchased from Peptides International. Endotoxin content for the stimulants used was $0.007 \text{ EU } (\mu\text{g hBD2})^{-1}$ and $>3 \times 10^7 \text{ EU } (\mu\text{g FnCW})^{-1}$ determined by limulus pyrochrome assay (Associates of Cape Cod).

Human primary GEC culture and stimulation. Healthy human gingival tissue samples were obtained from patients undergoing third-molar extraction at the Oral Surgery Clinic, School of Dentistry, University of Washington, in accordance with the University of

Washington Institutional Review Board approved procedures. Primary GECs were isolated and grown in keratinocyte basal medium with supplements as previously described (Chung & Dale, 2004; Krisanaprakornkit *et al.*, 1998, 2000). These primary cultures provide a physiologically appropriate target to examine bacterial responses. In consideration of individual variation due to genetic or other factors, epithelial cells from three donors were tested in duplicate or triplicate. Cells from additional donors were used for confirmatory studies. For all experiments, the fourth passage epithelial cells were stimulated when cells were at 80 % confluence. The cells were treated with either FnCW ($10 \mu\text{g ml}^{-1}$), or *P. gingivalis* cell wall (PgCW) extract ($1 \mu\text{g ml}^{-1}$) or hBD2 peptide ($2 \mu\text{g ml}^{-1}$) for the indicated time and harvested by scraping. The concentration of PgCW extract used in this study was the maximum that was non-toxic to GECs. To avoid effects of changes in epithelial cell physiology during their growth in culture, each time point had a corresponding untreated, control group. Each experimental condition was conducted in duplicate or triplicate.

RNA isolation, purification and quantitative real-time PCR (QRT-PCR). Total RNA was isolated from GECs with TRIzol and purified using the RNeasy mini kit (Qiagen) by standard procedures. The quality and integrity of the total RNA was assayed on an Agilent 2100 Bioanalyser. cDNA was prepared from $2 \mu\text{g}$ total RNA by standard techniques.

QRT-PCR was conducted using an aliquot of cDNA with Brilliant SYBR Green QPCR master mix (Stratagene). The amplification conditions were initial denaturation at 95°C for 12 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at $57\text{--}65^\circ\text{C}$ for 30 s and elongation at 72°C for 60 s. Melt-curve analysis was performed to confirm that the signal was that of the expected amplification product and not possible primer-dimers. Oligonucleotide primers were designed according to the published sequences or using Primer 3.0 software and UCSC Genome Bioinformatics (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi>; <http://genome.ucsc.edu/>) and are listed in Table 1. In initial experiments, amplification efficiency was determined for all primer pairs. QRT-PCR was performed in duplicate and normalized to the housekeeping gene ribosomal phosphoprotein (RPO). Results are expressed as the relative fold increase of stimulated samples over controls, referred to as Pfaffl's method (Pfaffl, 2001).

Detection of cytokines or protease inhibitors in culture supernatants. Human Cytokine Array III (Ray Biotech) was prepared as described by the manufacturer and used as a tool to detect multiple cytokines in cell culture supernatants. Human SLPI and pre-elafin/SKALP were quantified by sandwich ELISA (R&D Systems; HyCult

Table 1. Oligonucleotide primer sequences for real-time PCR

Gene name (accession no.)	Forward primer (5'–3')	Reverse primer (5'–3')	T_m (°C)	Product size (bp)
SLPI (NM_003064)	CCAGGGAAGAAGAGATGTTG	GGCTTCCTCCTTGTGGG	55	176
SKALP (NM_002638)	AGCAGCTTCTTGATCGTGGT	GACCTTGACTGGCTCTTGC	56	193
SERPINB1 (NM_030666)	TTCCTGGCGTTGAGTGAGAAC	TGTGCTGCCGTGTTACCTC	58	178
SERPINB2 (NM_002573)	CAGCACCGAAGACCAGATGG	CCTGCAAAATCGCATCAGGATAA	58	156
SERPINB3 (NM_006919)	GCTGAAGATCGCCAACAAG	CCCACTGCCCTTTGAAATAG	58	260
SERPINB4 (NM_002974)	TCAGTGAAGCCAACACCAAG	TGTTGCAGCTTTTCTGTGG	58	206
MMP1 (NM_002421)	AGTAGCTCAGATGACATTGATG	GCCGATGGGCTGGACAG	55	74
IL8 (NM_000584)	GAATGGGTTTGCTAGAATGTGATA	CAGACTAGGGTTGCCAGATTAAAC	56	91
CCL-20 (NM_004591)	TTTATTGTGGGCTTCACACG	GATTTGCGCACACAGACAAC	60	97
CXCL2 (GRO β) (NM_002089)	TGCCAGTGCTTGCAGAC	TCTTAACCATGGGCGATGC	58	157

Biotechnology). Samples were analysed in duplicate following the manufacturers' protocol.

Oligonucleotide microarrays. The RNA samples were processed for microarrays at the Center for Expression Array at the University of Washington following the manufacturer's protocols for determination of gene expression using GeneChip expression analysis (Affymetrix). Preparation of the biotinylated cRNA probe was performed as described elsewhere (Affymetrix GeneChip expression analysis manual) and used for hybridization onto HG-U133A GeneChips containing 22 283 different probe sets corresponding to 14 239 genes. Three independent biological samples were processed per treatment. To compare data from multiple experiments, the signal of each probe array was scaled to the same target intensity value by using GeneChip operating software (GCOS v1.4).

Statistical analysis and comparisons. Affymetrix array data were analysed by GCOS (Affymetrix). These raw values were then imported to BRB ArrayTools v3.0 for further analysis (Baldi & Long, 2001; Wright & Simon, 2003). A log base 2 transformation was applied to the data and each array was normalized by using the median intensity over the entire array. ArrayTools was used for processing expression data from multiple arrays, visualization of data, multidimensional scaling and clustering of genes and arrays. Significantly changed genes across treated groups were selected based on ANOVA at $P < 0.005$ (de Hoon *et al.*, 2004; Wright & Simon, 2003). Probe sets with mean expression value < 40 (near the limits of detection) were eliminated from further consideration.

For hierarchical clustering of significantly altered genes, log 2 transformed ratios were used as input into TIGR MultiExperiment Viewer (Saeed *et al.*, 2003). Hierarchical clustering analysis was conducted using mean linkage and Euclidean dissimilarity (Eisen *et al.*, 1998), and the fold change indicated colorimetrically.

Gene ontology (GO) and functional analysis. Evidence of co-regulation of multiple genes that are related by functional categories or pathways was obtained using GO for biological processes, cellular components and molecular functions (<http://www.geneontology.org>) (Ashburner *et al.*, 2000; Harris *et al.*, 2004). Genes that were significantly up-regulated (≥ 1.5 -fold and $P < 0.005$) or down-regulated (≤ 0.67 -fold and $P < 0.005$) were annotated with GO information with the MAPPFinder 2.0 program (<http://www.genmapp.org>) (Doniger *et al.*, 2003). For a GO term to be included in the table, at least four genes were changed significantly (nested results) and the Z-score was above 4. Functional pathway analysis of the significantly altered genes was conducted using FatiGO (Fast Assignment and Transference of Information using GO) (<http://www.fatigo.org>) (Al-Shahrour *et al.*, 2005). The genes for the cross-plot were selected based on significant expression in FnCW- and hBD2-stimulated cells from two donors. The cross-plot was generated by JMP 6 statistical software (SAS).

RESULTS AND DISCUSSION

Alteration of global gene expression induced by FnCW

Global gene expression of primary human GECs treated with FnCW extract ($10 \mu\text{g ml}^{-1}$) was analysed using Affymetrix HG-U133A microarrays. By using a cell wall preparation of *F. nucleatum*, we had a reproducible stimulant, and eliminated potentially confounding effects due to bacterial growth during the stimulation period (Whitney *et al.*, 2003) and possible effects due to bacterial

invasion of cells. Class comparison analysis revealed that 829 genes were significantly changed ($P < 0.005$, ANOVA). Agglomerative hierarchical clustering of the 829 unique probes (Fig. 1a, Supplementary Table S1 available with the online journal) provides an overview of altered GEC gene expression in response to FnCW. Among them, 581 genes were significantly down-regulated, while 248 genes were significantly up-regulated. The genes shown in Fig. 1 were consistently regulated by FnCW in cells from three donors, although the response amplitudes for specific genes varied between donors (for example, Fig. 1b).

The 20 most highly up-regulated genes included CCL20, S100A7, SKALP, IL1F9, IL8, CXCL5, C3, IL32, SAA1, SPRR2C and CXCL1 ($P < 0.005$). Fourteen out of twenty were cytokines, innate immune or inflammatory markers, antimicrobials, or protease inhibitors, while two additional strongly up-regulated genes (SPRR2B, SPRR2C, small proline-rich proteins) are related to structural aspects of the epithelial barrier. Many of these individual genes are shown in Fig. 1(b, c). The most obvious down-regulated genes (Fig. 1d) included cell cycle regulatory genes (CDC20, SKP2, PCNA, POLE2) and ubiquitin-proteasome-associated genes (UBAP2L, PSMD11).

The broad biological themes revealed by the clusters of induced and repressed genes were independently supported by functional analysis (GO analysis, Fig. 1e). The up-regulated biological processes included immune responses, defence responses, chemotaxis and wound healing, cytokine activity and protease inhibition, while down-regulated processes included mitosis, cell division and ubiquitin-dependent protein degradation. Pathway analysis by FatiGO (Al-Shahrour *et al.*, 2005) confirmed cytokine/chemokine receptor interaction, calcium-mediated signalling, Jak-STAT signalling, WNT signalling, Toll-like receptor signalling pathways and a decrease in cell cycle and proliferation (not shown). These overall results are similar to those reported by Hasegawa *et al.* (2007) for *F. nucleatum* co-cultured with immortalized oral epithelial cells for a 2 h period. However, our results showed less effect on transcriptional regulation of MAPK signalling at the longer time analysed here (24 h) with our cell wall stimulus and primary oral epithelial cells.

Comparison of gene expression altered by FnCW and hBD2

The antimicrobial peptide hBD2 is expressed in normal uninfamed gingival tissue and is up-regulated in GECs by commensal bacteria (Dale *et al.*, 2001; Krisanaprakornkit *et al.*, 2000). In order to investigate the immunomodulatory effects of hBD2 and to determine if part of the response to FnCW might be due to the presence of hBD2, we compared gene expression of GECs stimulated with FnCW or hBD2 using GECs from two donors. FnCW and hBD2 induced many gene changes in the same GO categories, including defence response, cell cycle, chemotaxis, ubiquitin, differentiation and signal transduction.

Fig. 2. Cross-plot comparison of genes significantly altered in GECs treated with FnCW or hBD2 for 24 h. The plot represents log-transformed fold change of FnCW (y-axis) versus log-transformed fold change of hBD2 (x-axis) stimulated gene expression (at least 1.5-fold; $P < 0.01$). The colour indicates the GO category; black squares represent other genes. Note that genes that lie within the upper right and lower left quadrants represent those with common regulation by FnCW and hBD2, while genes that lie near the axes represent genes whose expression is altered only by FnCW or by hBD2. Only a few genes show divergent regulation and are found in the upper left and lower right quadrants.

However, the detailed gene lists for each GO category were different for FnCW and hBD2 treatments. The cross-plot illustrates the comparison of 595 genes significantly modulated ($P < 0.01$) by FnCW and hBD2 (Fig. 2). The common genes up-regulated included cytokines and chemokines (IL1 α , IL1 β , IL8, CSF2, CXCL1), and genes related to antimicrobial function (S100A7, CCL20) and NF- κ B signalling (TNFAIP3). The common down-regulated genes included cell cycle-related genes (GTSE1, CDCA3 and CDC20) and ubiquitin-related genes (USB2C, PSMD11). These results suggest that many transcriptional responses to commensal bacteria in the tissue could be due to the presence of hBD2. However, expressions of several genes for protease inhibitors (SERPINB2, 4) were uniquely up-regulated by FnCW. In contrast, interferon-related genes (IFI27, IRF4) and growth factor-related genes (TGFB1, TGFBR2) were up-regulated by hBD2. Both stimuli up-regulated genes associated with physical aspects of the epithelial barrier; FnCW up-regulated SPRR2C, associated with the cornified cell envelope (Gibbs *et al.*, 1993), and hBD2 up-regulated corneodesmosin (CDSN), a protein that mediates adhesion and desquamation of stratified epithelial cells (Jonca *et al.*, 2002), as well as cathepsin B (CTSB), a protease that functions in the activation of CCL20 (Hasan *et al.*, 2006). Thus, while similar categories of genes responded to the two stimuli, the specific responses differ and the physiological response to the commensal is probably largely independent of hBD2 in the tissue.

Regulation of innate immunity and inflammation responses

Genes up-regulated by FnCW included those encoding antimicrobial peptides and proteins (Table 2). Additional genes of defence responses included chemokines IL8,

CXCL1, CXCL3, CXCL5 and CXCL10, which attract neutrophils, monocytes and macrophages, or lymphocytes and CSF2 and CSF3 that stimulate neutrophil development (Inano *et al.*, 1998; Wolach *et al.*, 2000). CCL20 and hBD2 both attract dendritic cells in addition to their antimicrobial function (Greaves *et al.*, 1997; Yang *et al.*, 1999). Proinflammatory cytokines IL1 α and IL1 β were up-regulated, as well as ICAM1/CD54. FnCW also induced genes for interleukin receptors (IL13RA2, IL17RB) and other G-protein coupled receptors (GPR109B), the IL1 antagonist (IL1RN) and the interferon receptor (IFNGR1). FnCW up-regulated the genes of tissue plasminogen activator (PLAT) and its receptor (PLAUR), which have a role in innate and adaptive immunity through promoting cell migration, adhesion and participating in T-cell priming (Mondino & Blasi, 2004). Many of these genes were up-regulated at 6 h suggesting a rapid and in some cases transient response (Table 2). The transient up-regulation of gene expression may be due to post-transcriptional regulation of mRNA stability, for example, via sequences in the 3' untranslated region of cytokine and chemokine mRNAs (Fan *et al.*, 2005).

Induction of protease inhibitors

Up-regulation of the group of neutrophil chemoattractants is consistent with the normal flow of neutrophils into the space between the tooth and soft tissue due to the cytokine gradient within the normal epithelium. Neutrophils are part of the continuous surveillance of the gingival sulcus (Tonetti *et al.*, 1998). However, proteases released by neutrophils contribute to inflammation and tissue damage. We found that multiple protease inhibitors were strongly up-regulated in response to FnCW but not to hBD2 (Table 3). These inhibitors are expected to target proteases released by neutrophils and therefore control potential

Fig. 3. Markers of innate immunity induced by FnCW and hBD2 in GECs. GECs from two donors were treated with hBD2 peptide ($2 \mu\text{g ml}^{-1}$) or FnCW extract ($10 \mu\text{g ml}^{-1}$) for 24 h. The relative change in expression of mRNAs for hBD2, IL8, CCL20, CXCL2 and MMP1 was evaluated by QRT-PCR. Results are shown as mean fold change $\pm$ SEM over unstimulated controls. The data represent the mean of two independent experiments performed in duplicate.

Fig. 4. Expression of protease inhibitors by GECs in response to FnCW and PgCW. GECs from three different donors were treated with FnCW ($10 \mu\text{g ml}^{-1}$) or PgCW ($1 \mu\text{g ml}^{-1}$) extract for 24 h and evaluated for mRNA and protein. (a) Comparison of mRNA expression in GECs measured by QRT-PCR. Results shown are the mean fold change $\pm$ SD. Values that are significantly higher in FnCW-treated cells (hatched bars) than PgCW-treated cells (black bars) are indicated by * ($P < 0.05$) and ** ($P < 0.01$). (b) Protease inhibitor secretion in culture supernatants quantified by ELISA. Results shown are the mean percentage induction $\pm$ SD from cells of three different donors. The secretion of SLPI and elafin/SKALP was significantly higher in FnCW-treated cells (hatched bars) than that PgCW-treated cells (black bars) compared to unstimulated controls (white bars). Significant difference from controls are shown by * ($P < 0.05$) and ** ($P < 0.01$).

tissue damage (Magert *et al.*, 2005), and represent a protective response in the presence of commensal bacteria.

Protease inhibitors specific for both serine (SKALP, SLPI, SERPIN1, 2, 4) and cysteine proteases (SERPINB1, 3, 4, CSTB) were up-regulated; some these are associated with innate host defence in other tissues (Magert *et al.*, 2005; Zhu *et al.*, 2002). For example, both SLPI and SKALP have antimicrobial activity against Gram-positive and Gram-negative pathogens (McMichael *et al.*, 2005; Sallenave *et al.*, 2003). Expression of these genes increased significantly at both 6 and 24 h in cells treated with FnCW. SERPINB3 and B4 were among the most highly expressed genes at 6 h (Table 3).

In addition to providing protection against neutrophil proteases, these protease inhibitors may protect against bacterial proteases secreted by pathogens. Three well-characterized periodontal pathogens, *P. gingivalis*, *Treponema denticola* and *Tannerella forsythensis*, each have serine or cysteine proteases that are important virulence factors (Curtis *et al.*, 2001; Fenno *et al.*, 2001; Saito *et al.*, 1997; van der Reijden *et al.*, 2006). The cysteine proteases (gingipains) of *P. gingivalis* stimulate protease-activated receptors (Chung *et al.*, 2004; Loubakos *et al.*, 2001; Uehara *et al.*, 2002) and this family of receptors has been implicated in periodontal disease (Holzhausen *et al.*, 2005, 2006). Thus, up-regulation of protease inhibitor genes by commensal bacteria may specifically block effects of pathogenic bacteria, such as *P. gingivalis*, as well as limiting inflammatory tissue damage caused by neutrophil proteases.

Validation of microarray findings

Up-regulation of both cytokines and protease inhibitors was verified by other assays. We evaluated multiple cytokine and chemokine mRNAs by QRT-PCR relative to unstimulated controls. The trend of expression level was consistent with the microarray data. HBD2 slightly induced these selected genes, while FnCW greatly induced the genes tested (Fig. 3). Cytokine and chemokine protein levels secreted into cell culture supernatants were evaluated by semiquantitative protein arrays (Table 4). Again, results for FnCW-treated GECs were consistent with microarray data except for IL1 α and IL1 β , which are poorly secreted by epithelial cells (Mizutani *et al.*, 1991). Our results on up-regulation of cytokines and chemokines are consistent with those of others (Hasegawa *et al.*, 2007), for example IL6 and IL8. For comparison, we tested secreted cytokine levels in GECs treated with a PgCW preparation; these cells had very low levels of nearly all of the cytokines and chemokines evaluated, although the two donors showed some variation (data not shown).

The expression of mRNA for six protease inhibitors was also evaluated by QRT-PCR (Fig. 4a). Significant up-regulation of protease inhibitors was seen in cells stimulated with FnCW. Although there were variations between cells from different donors, the mean fold change

Table 2. Gene expression regulated by FnCW in GECs

Name	Gene symbol	Affymetrix array		
		Fold change at 6 h	Fold change at 24 h	Parametric P value at 24 h
Antimicrobial effectors				
Chemokine (C-C motif) ligand 20	CCL20	72.7	21.7	<1e-07
S100 A7 (psoriasin 1)	S100A7	1.3	16.8	1.09E-05
Complement component 3	C3	1.0	7.5	1.17E-05
S100 A9 (calgranulin B)	S100A9	4.8	6.3	0.003
S100 A8 (calgranulin A)	S100A8	3.1	3.6	0.015
S100 A12 (calgranulin C)	S100A12	2.8	3.0	0.004
β -defensin-2	DEFB4	2.1	2.6	0.002
Decay accelerating factor (CD55)	DAF	1.1	1.5	0.001
Chemokine, cytokines and receptors				
Interleukin 8	IL8	27.8	9.5	1.57E-05
Chemokine (C-X-C motif) ligand 5 (ENA-78)	CXCL5	40.5	8.9	0.000
Chemokine (C-C motif) ligand 5	CCL5	5.0	2.7	0.002
Chemokine (C-X-C motif) ligand 1	CXCL1	8.9	5.1	0.000
Chemokine (C-X-C motif) ligand 2	CXCL2	6.8	2.7	0.000
Chemokine (C-X-C motif) ligand 10	CXCL10	3.4	1.9	0.000
Colony stimulating factor 2	CSF2	4.3	4.8	0.000
Colony stimulating factor 3	CSF3	3.6	3.5	5.55E-05
Interleukin 1 family, member 9	IL1F9	24.9	10.2	1.40E-06
Interleukin 13 receptor alpha 2	IL13RA2	5.1	3.1	0.001
Interleukin 1 beta	IL1B	4.5	2.5	6.00E-07
Interleukin 1 receptor antagonist	IL1RN	8.1	2.2	0.002
Interleukin 6 (beta interferon 2)	IL6	3.2	2.1	0.001
Heparin-binding EGF-like growth factor	HBEGF	1.8	2.4	2.25E-05
Interleukin 19	IL19	2.0	1.9	0.005
Interleukin 17 receptor B	IL17RB	1.3	1.8	0.005
Interleukin 1 alpha	IL1A	2.7	1.7	5.70E-06
Interleukin enhancer-binding factor	ILF3	0.7	0.6	0.002
TNFAIP3-interacting protein 1	TNIP1	3.8	2.2	7.80E-06
TNF- α -induced protein 3	TNFAIP3	5.5	2.5	0.001
TNF- α -induced protein 2	TNFAIP2	10.8	1.9	0.000
Ubiquitination/degradation				
Ubiquitin-conjugating enzyme E2C	UBE2C	0.9	0.7	0.005
Proteasome subunit alpha type 2	PSMA2	0.9	0.7	0.005
Ubiquitin-specific peptidase 4	USP4	0.8	0.9	0.001
Ubiquitin-conjugating enzyme E2L 3	UBE2L3	0.9	0.8	0.004
Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	UCHL3	0.9	0.8	0.005
Ubiquitin-specific peptidase 48	USP48	0.8	0.8	0.001
Ubiquitin protein ligase E3A	UBE3A	1.0	0.8	0.001
Anaphase-promoting complex subunit 5	ANAPC5	0.8	0.8	0.001
Ubiquitin-specific peptidase 1	USP1	0.7	0.8	0.002
Ubiquitin fusion degradation 1 like	UFD1L	0.9	0.8	0.003
Ubiquitin-conjugating enzyme E2N	UBE2N	1.0	0.8	0.000
Cell cycle				
Cell division cycle 20 homologue	CDC20	0.8	0.5	0.000
Cell division cycle 45-like	CDC45L	0.3	0.6	0.000
Extra spindle poles like 1	ESPL1	0.8	0.7	0.004
S-phase kinase-associated protein 2 (p45)	SKP2	0.5	0.5	1.00E-07
Nucleolar and spindle-associated protein 1	NUSAP1	0.7	0.6	0.000
Spindle pole body component 25 homologue	SPBC25	0.8	0.7	0.003
Others				
Matrix metallopeptidase 1	MMP1	3.8	4.3	0.001
Matrix metallopeptidase 9	MMP9	1.1	4.3	1.70E-06
Matrix metallopeptidase 10	MMP10	4.2	3.5	5.80E-06

Table 2. cont.

Name	Gene symbol	Affymetrix array		
		Fold change at 6 h	Fold change at 24 h	Parametric P value at 24 h
CASP8 and FADD-like apoptosis regulator	CFLAR	5.2	1.7	0.002
Plasminogen activator, tissue	PLAT	3.2	1.7	4.31E-05
Plasminogen activator, urokinase receptor	PLAUR	4.0	1.8	0.002
Serum amyloid A1	SAA1	2.4	5.7	0.001
Serum amyloid A1/A2	SAA1/SAA2	1.8	4.6	0.001
NDRG family member 4	NDRG4	2.4	4.5	8.30E-06
NF- κ B inhibitor alpha	NFKBIA	2.6	2.2	2.16E-04
Single immunoglobulin IL1R related	SIGIRR	1.1	1.5	0.012

for these protease inhibitors was from 2.5- to over 30-fold with FnCW, while PgCW did not result in up-regulation of mRNAs of the protease inhibitors tested. SLPI and elafin/SKALP protein concentrations in culture supernatants were determined by ELISA (Fig. 4b). The secretion of both SLPI and elafin/SKALP were highly increased with exposure to FnCW (SLPI 215.3%; elafin/SKALP 625.5%), while slightly decreased or not changed with exposure to PgCW (Fig. 4b), reflecting the QRT-PCR results. Thus, this response seems to be specific for the commensal. The poor cellular response to *P. gingivalis* is consistent with the

'stealth-like' action of this pathogen (Darveau *et al.*, 1998). Live bacterial cultures of *F. nucleatum* and the cell wall preparation had similar levels of up-regulation of these protease inhibitors (data not shown). Nevertheless, live cultures of *P. gingivalis* may have different effects on epithelial cells because of the ability of *P. gingivalis* to invade epithelial cells (Lamont *et al.*, 1995), and due to presence of additional virulence factors such as proteases (Curtis *et al.*, 2001) and fimbriae (Handfield *et al.*, 2005; Zhou & Amar, 2006). For example, live *P. gingivalis* results in up-regulation of multiple cytokines and chemokines, CCL20, and hBD2

Table 3. Protease inhibitors induced in GECs

Gene symbol	Other name	Protease inhibited	Potential function	Fold change		
				FnCW (10 μ g ml ⁻¹)		hBD2 (2 μ g ml ⁻¹) (24 h)
				6 h	24 h	
PI3	Elafin, SKALP, trappin	Elastase, PMN proteinase 3	Innate immunity, differentiation, blocks TNF α processing, anti-microbial	2.95/1.5*	14.31/5.8*	1.9/1.5*
SERPINB1	EIA, ELANH2	Elastase, cathepsin G, proteinase 2	Innate immunity, inhibits PMN proteases to limit inflammatory damage	2.1	3.2	1.1
SERPINB2	PAI2	Thrombin, urokinase plasminogen activator	Regulates extravascular plasminogen activation	4.8	2.2	0.9
SERPINB3	SCCA1	Cathepsin S, K, L, papain	Inhibits cathepsin S, K, L and modulates host immune response	92.0/14.2*	19.4/4.5*	1.9/1.3*
SERPINB4	SCCA2, leupin	Cathepsin G, mast cell chymase	Inhibits mast cell proteases and limits damage	239.2/39.0*	8.6/13.2*	1.2/1.1*
SLPI		Elastase, cathepsin B, trypsin	Stimulates wound healing, innate immunity, inhibits PMN proteases, antimicrobial	1.9	4.3	1.6
CSTB	Cystatin B	StefinB	Protection against intracellular proteases leaking from lysosomes	1.2	2.0	1

*Fold change is indicated by two Affymetrix probe sets.

(Kinane *et al.*, 2006); this may occur via secreted proteases (Chung *et al.*, 2004) that are not active in our PgCW preparation. In addition, epithelial cell responses may show individual variation, for example, cytokine responses to *P. gingivalis* have been associated with TLR4 polymorphisms (Kinane *et al.*, 2006).

Effects on genes that regulate NF- κ B function

NF- κ B is a critical transcription factor involved in inflammatory responses such as IL8 up-regulation as seen here. However, multiple genes that reduce NF- κ B function were up-regulated at both 6 and 24 h stimulation with FnCW (Table 2). These included NFKBIA (2.6-fold at 6 h) encoding the I κ B α subunit, and TNFAIP3/A20 (5.5-fold) and CFLAR (5.2-fold), which both function to limit signalling by NF- κ B (Banno *et al.*, 2005; Gon *et al.*, 2004; Wertz *et al.*, 2004). NFKBIA, TNFAIP3/A20 and CFLAR were also up-regulated in an immortalized oral epithelial cell line by pathogenic bacteria and this was related to altered apoptosis (Handfield *et al.*, 2005). However, with the commensal stimulus used here, these changes are coupled with long-term up-regulation of SIGIRR (single immunoglobulin IL1R related) (1.5-fold, 24 h), which limits Toll-like receptor-mediated inflammatory responses (Qin *et al.*, 2005; Wald *et al.*, 2003) (Table 2). In addition, we observed the persistent decrease in multiple genes of the ubiquitinylation/proteasome pathway. This adds another mechanism to limit NF- κ B signalling by stabilizing I- κ B and sequestering the transcription factor in the cytoplasm. Thirteen genes involved in the protein ubiquitination/proteasome degradation process were down-regulated after GECs were treated with FnCW for 24 h. Some of these genes were also down-regulated at 6 h, for example USP1 (ubiquitin-specific peptidase) (Table 2). Several of these

mechanisms to suppress NF- κ B function have been identified in other epithelial tissues or animal models. TNFAIP3/A20 is a ubiquitin ligase that is associated with RIP (receptor-interacting protein) in airway epithelial cells and blocks tumour necrosis factor (TNF)-induced NF- κ B signalling (Ferran *et al.*, 1998; Gon *et al.*, 2004; Wertz *et al.*, 2004). CFLAR (CASH, CASP8 and FADD-like apoptosis regulator) inhibits NF- κ B by binding to TRAF-1 and TRAF-2, and blocks transmission of signals from the receptor in epidermal keratinocytes (Banno *et al.*, 2005; Irmeler *et al.*, 1997). SIGIRR suppresses activation of IL1 receptor-associated kinase (IRAK) in mice (Qin *et al.*, 2005; Wald *et al.*, 2003). Down-regulation of ubiquitin-related genes, as seen here, also contributes to suppression of inflammatory signals in intestinal epithelial cells in response to non-pathogenic *Salmonella* spp. (Neish *et al.*, 2000) and *Lactobacillus casei* (Tien *et al.*, 2006). Mechanisms that allow epithelial cells to distinguish commensal and pathogenic bacteria are physiologically important in the gut (Ismail & Hooper, 2005; Kelly *et al.*, 2004; Neish *et al.*, 2000; Rakoff-Nahoum *et al.*, 2004), as well as in the oral cavity. Disruption of these responses due to mutations in NOD2 (nucleotide-binding oligomerization domain) is associated with Crohn's disease (Ogura *et al.*, 2001; Strober *et al.*, 2006). Our results suggest that the commensal stimulus has the overall effect of suppressing NF- κ B function in oral epithelia, which normally maintain a non-inflamed condition. Our observations suggest that the non-pathogen, *F. nucleatum*, limits NF- κ B-mediated signalling by multiple mechanisms and that these changes may reduce expression of inflammatory products in oral epithelia. Although we show up-regulation of proinflammatory cytokines and chemokines, especially at short exposure times to FnCW, the long-term effects are likely to be balanced by reduced NF- κ B signalling.

Table 4. Secretion of cytokines/chemokines by GECs

Name	Gene symbol	Fold change for treatment*	
		FnCW	PgCW
Interleukin 1 alpha	IL1A	1.2 $\pm$ 0.01	0.03 $\pm$ 0.00
Interleukin 1 beta	IL1B	1.3 $\pm$ 0.04	0.05 $\pm$ 0.00
Interleukin 6 (beta interferon 2)	IL6	113.3 $\pm$ 0.00†	0.0 $\pm$ 0.00
Interleukin 8	IL8	1.8 $\pm$ 0.06‡	0.65 $\pm$ 0.06
Chemokine (C-X-C motif) ligand 1 (GRO α)	CXCL1	1.3 $\pm$ 0.02	0.14 $\pm$ 0.00
Chemokine (C-X-C motif) ligand 2 (GRO β)	CXCL2	9.6 $\pm$ 0.00	0.0 $\pm$ 0.00
Colony stimulating factor 2 (GM-CSF)	CSF2	2.0 $\pm$ 0.01	0.9 $\pm$ 0.05
Chemokine (C-X-C motif) ligand 5 (ENA-78)	CXCL5	2.8 $\pm$ 0.00	0.0 $\pm$ 0.00
Chemokine (C-C motif) ligand 5 (RANTES)	CCL5	1.5 $\pm$ 0.01	0.01 $\pm$ 0.00

*Fold change was calculated from the density of the protein array spots in treated versus control samples after normalization to positive control proteins. Results represent two independent experiments using GECs from different donors evaluated in duplicate. The densities were measured by the Alpha Ease FC software version 4.1.0 (Alpha Innotech).

†This cytokine was present in the treated sample, but essentially absent in the control.

‡IL8 value is a minimum due to saturation of the signal in the FnCW-treated sample.

Overall findings

Our results show that *F. nucleatum* not only induces the antimicrobial peptide hBD2, as shown in previous studies, but also influences immune responses through the induction of cytokines and chemokines, and apparent suppression of NF- κ B function. Further, *F. nucleatum* aids in the maintenance of a healthy mucosal surface by increasing transcription of a group of protease inhibitors whose translation as active inhibitors may block tissue damage by proteases from neutrophils, which are continually migrating into the oral cavity via the gingival sulcus. The ability of these inhibitors to block proteases secreted by pathogens is currently under study. We show that hBD2 acts as a cytokine, and that both hBD2 and *F. nucleatum* may enhance the epithelial barrier as indicated by transcription of differentiation related products, such as corneodesmosin and small proline rich proteins, as well as antimicrobial peptides. We propose that these protective responses are biologically significant in normal gingival tissue in view of the fact that commensals and bridge organisms, such as *F. nucleatum*, colonize the epithelium within the gingival sulcus prior to pathogens such as *P. gingivalis*, therefore, protective epithelial cell responses that are induced by *F. nucleatum* would occur prior to exposure to *P. gingivalis* and could delay the onset or the severity of the effects of *P. gingivalis* infection. Although the epithelial innate immune responses cannot be expected to prevent *P. gingivalis* infection they may defer the infection and reduce its intensity.

These observations support the hypothesis that normal oral epithelial cells, in the presence of commensal organisms and hBD2, are primed for response to subsequent exposure to oral pathogens. They contribute to our understanding of oral health and may provide ideas for new therapeutic means to control chronic periodontal disease.

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