Porphyromonas gingivalis enhances FasL expression via up-regulation of NF κ B-mediated gene transcription and induces apoptotic cell death in human gingival epithelial cells

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The interaction between epithelial cells and micro-organisms is often a crucial initiating event in infectious diseases. Infection with *Porphyromonas gingivalis*, a Gram-negative anaerobe, is strongly associated with severe periodontal disease. This bacterium possesses an array of virulence factors, some of which can induce apoptosis. The tumour necrosis factor (TNF) receptor family is involved in the regulation of cellular homeostasis, cell surface molecules involved in phagocytosis, Fas ligand (L) expression and activation of the caspase cascade resulting in DNA fragmentation and cell blebbing. The current study examined the role of nuclear factor- κ B (NF κ B) in FasL-mediated apoptotic cell death in primary human gingival epithelial cells (HGEC) induced by heat-killed *P. gingivalis*, probably through TLR signalling pathways. A marked up-regulation of TLR2 and Fas–FasL was detected in HGEC stimulated with *P. gingivalis*. Activation of NF κ B by *P. gingivalis* in HGEC was demonstrated by an NF κ B promoter luciferase assay as well as by phosphorylation of p65 as detected by Western blotting. Activation of cleaved caspase-3 and caspase-8 resulted in apoptotic cell death of HGEC. The survival proteins c-IAP-1/c-IAP-2 were decreased in HGEC exposed to *P. gingivalis*. HGEC apoptosis induced by *P. gingivalis* was inhibited by an anti-human FasL monoclonal antibody. Blockade of NF κ B by helenalin resulted in down-regulation of FasL whereas a caspase-8 inhibitor did not decrease FasL. Taken together, these studies show that *P. gingivalis* can induce epithelial cell apoptosis through Fas–FasL up-regulation and activation of caspase-3 and caspase-8.

Received 26 August 2005

Revised 17 November 2005

Accepted 18 November 2005

INTRODUCTION

Periodontal disease comprises a group of chronic inflammatory disorders of the gingiva and periodontium that in severe cases, periodontitis, results in tooth loss in adults (Offenbacher, 1996). Many basic and clinical studies

support the concept that oral microbes trigger the disease but periodontal tissue destruction results from host-mediated injury due to continuous stimulation with bacterial components and products. Numerous studies have examined the cytokine response of periodontal tissues upon exposure to periodontopathic bacteria. It has been shown that *Porphyromonas gingivalis*, a Gram-negative bacterium, is strongly associated with the onset of adult periodontitis. *P. gingivalis* induces interleukin (IL)-1 β , tumour necrosis factor (TNF)- α , IL-6 and IL-8 in the KB-cell line, a fibroblast cell line, as well as in human primary gingival epithelial cells (HGEC) (Roberts *et al.*, 1997; Sandros *et al.*, 2000). The precise mechanism by which *P. gingivalis* activates oral epithelial cells to produce proinflammatory cytokines and cause inflammation has not been elucidated. Recent studies have implicated Toll-like receptors (TLR) in bacterial

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Abbreviations: DISC, death-inducing signalling complex; EMSA, electrophoretic mobility shift assay; FADD, Fas-associated death domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGEC, human gingival epithelial cells; IL, interleukin; LDH, lactate dehydrogenase; NF κ B, nuclear factor- κ B; OMP, outer-membrane protein; TLR, Toll-like receptor; TNF, tumour necrosis factor.

signalling, which trigger an intracellular cascade that leads to activation of the transcription factor nuclear factor- κ B (NF κ B). *In vitro* studies with primary gingival epithelial cells have shown expression of TLR1, TLR2 and TLR6. However, only TLR2 and TLR4 have been detected in gingival tissue from patients with periodontitis (Asai *et al.*, 2001, 2003; Yoshimura *et al.*, 2002, 2003).

Epithelial cells act as the first barrier to bacterial invasion, and the interaction between *P. gingivalis* and epithelial cells is likely to be an important part of the pathogenesis of periodontal disease. *P. gingivalis* is primarily detected in deep periodontal pockets, especially in active inflammatory sites. At this location, significant bacterial death has been detected. In addition, morphological studies have detected epithelial cell death *in situ* (Tonetti *et al.*, 1998). It has been suggested that p53 expression associated with DNA damage is a prevalent phenomenon in chronically inflamed human gingiva and that apoptosis may be important for the maintenance of local immune homeostasis in the gingival tissue (Socransky & Haffajee, 1992). Although apoptosis induced by bacterial pathogens is important in many infectious diseases, it is unknown whether *P. gingivalis* causes the apoptosis observed in HGEC and, if so, the mechanisms involved (Chen & Zychlinsky, 1994).

The outcome of the interaction between *P. gingivalis* and HGEC is controversial. The results of Nakhjiri *et al.* (2001) have shown that up-regulation of Bcl-2 leads to inhibition of apoptosis in HGEC, and prevention of apoptosis may represent a strategy for *P. gingivalis* survival in HGEC. Apoptosis plays a critical role in the removal of damaged cells and regulates inflammation and the host immune response (Slots & Listgarten, 1988). Fas–FasL interactions represent a major apoptotic pathway that underlies many physiological and pathological causes of cell death (Nagata, 1997). FasL is also up-regulated by various stress stimuli and causes apoptotic death of lymphocytes but there are few reports directly examining FasL induction in periodontal disease.

Upon stimulation, Fas undergoes trimerization and recruits the pro-apoptotic adapter protein FADD (Fas-associated death domain) and procaspase-8 [FADD-like IL-1 β -converting enzyme (FLICE)], to form a death-inducing signalling complex (DISC) (Asai *et al.*, 2001; Boldin *et al.*, 1996; Chen & Zychlinsky, 1994; Chinnaiyan *et al.*, 1995; Lyss *et al.*, 1998). Recruitment of procaspase-8 to the DISC leads to its proteolytic activation to caspase-8, followed by initiation of a caspase cascade that leads to apoptosis (Medema *et al.*, 1997). Since Fas-mediated death of HGEC could play an important role in the pathogenesis of periodontal disease, the present work was performed to examine whether heat-killed *P. gingivalis* could induce apoptosis in HGEC and to elucidate the mechanisms involved in apoptotic cell death.

We now report that heat-killed *P. gingivalis* can induce FasL-dependent apoptotic cell death in HGEC through up-regulation of FasL gene expression and activation of

caspase-3 and -8. The *P. gingivalis*-induced FasL up-regulation was mediated by increased NF κ B gene transcription and activation of NF κ B. Given the specific up-regulation of TLR2, but not TLR4, that was observed, these studies suggest TLR2 could be implicated in the initiation of these pathways. These studies describe a mechanism whereby *P. gingivalis* can regulate epithelial cell survival with potential implications for mucosal barrier function and the downstream regulation of the immune response.

METHODS

Bacterial strains and conditions. *P. gingivalis* strain 33277 was kindly provided by Dr D. Demuth (University of Louisville, KY, USA). *P. gingivalis* was grown in trypticase soy broth (Becton Dickinson) supplemented with 1% yeast extract (Difco) and 1% each of menadione (1 mg ml⁻¹) and haemin (5 mg ml⁻¹) under anaerobic conditions (85% N₂, 10% CO₂ and 10% H₂; Coy Laboratory) for 2 days. After cultivation, the bacteria were harvested by centrifugation, washed three times in PBS (pH 7.4) and heat-inactivated for 1 h at 60 °C. *P. gingivalis* was resuspended in MCDB 153 medium (Sigma).

Preparation of cells. Healthy gingival tissues, which had been surgically dissected through the process of wisdom tooth extraction and which were to be discarded, were collected. HGEC were prepared from healthy gingival tissues taken from two donors according to a Human Studies protocol from the University of Louisville. Tissues were collected with the patient's informed consent. Gingival tissue was treated with 0.025% trypsin and 0.01% EDTA overnight at 4 °C and HGEC were isolated as previously described (Uchida *et al.*, 2001). The cell suspension was centrifuged at 120 g for 5 min, and the pellet was suspended in MCDB 153 medium (pH 7.4) (Sigma) containing 10 μ g insulin ml⁻¹, 5 μ g transferrin ml⁻¹, 10 μ M 2-mercaptoethanol, 10 μ M 2-aminoethanol and 10 nM sodium selenite supplemented with 50 μ g bovine pituitary extract ml⁻¹, 100 units penicillin ml⁻¹, 100 μ g streptomycin ml⁻¹ and 50 ng amphotericin ml⁻¹ (Uchida *et al.*, 2001). The cells were seeded in 60 mm plastic culture plates coated with type I collagen and incubated in 5% CO₂ at 37 °C. When the cells reached 80% of confluence, they were harvested and subcultured.

At the third or fourth day of cultures, HGEC were harvested, seeded at a density of 5×10^4 cells in six-well culture plates coated with type I collagen, and maintained in 2 ml medium. After 6 days, these cells were washed twice with MCDB 153 medium. Subsequently, heat-killed *P. gingivalis* (5×10^7), which was resuspended in MCDB 153 medium, was added for the indicated time periods before the end of the incubation on day 7. Furthermore, HGEC in cultures at the third or fourth passages were subjected to immunoblotting with anti-cytokeratin type I and II as previously described (data not shown) (Sugiyama *et al.*, 1996).

Cell viability. HGEC were plated onto six-well plates and treated with heat-inactivated *P. gingivalis*. The cells were harvested at 8 h, washed in cold PBS, treated with DNase-free RNase (50 mg ml⁻¹), stained with propidium iodide and subjected to flow cytometry, analysed on a Becton-Dickinson FACS-Scan. In addition, the cells were stained with trypan blue dye and counted by a method described elsewhere (McCloskey *et al.*, 1998). Measurements of lactate dehydrogenase (LDH) were performed. LDH release was measured by determining LDH activity (measured spectrophotometrically at 340 nm) in the medium and total cells by the method described

elsewhere (Lash *et al.*, 2001). LDH release (%) = [(LDH activity in medium)/(LDH activity in medium + LDH activity in total cells)] × 100.

Reverse transcription (RT)-PCR. RNA from each culture was extracted using TRIZOL (Invitrogen) and total cell RNA was purified according to the manufacturer's instructions and quantified. One microgram of total RNA extract was used to perform first-strand cDNA synthesis with a first-strand cDNA synthesis kit (Roche) in a total volume of 20 µl. One microlitre of each generated cDNA reaction was used as template DNA for the PCR using a High Fidelity Expand system (Roche). Primer sets for amplification of β -actin and TLR2 were used. Primer pairs for β -actin were purchased from R&D Systems. Primers for TLR2 were described elsewhere (5'-GAACTGCGAGATACTGATTTGGA-3'/5'-TTGGGAATGCAGCCTGTTACACT-3', 691 bp) (Kuroshima *et al.*, 2004). The RT-PCR products were analysed by electrophoresis in 2% agarose gels.

Real-time PCR. For real-time PCR, the first strand cDNA was synthesized using TaqMan Reverse transcription reagents (Applied Biosystems). The reverse transcription was carried out using 1 × TaqMan RT buffer, 5.5 mM MgCl₂, 500 µM each dNTP, 2.5 mM random hexamers, 8 U RNase inhibitor and 25 U Multiscribe Reverse Transcriptase with 200 ng total RNA. The cycling conditions were 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C. Reactions in which the enzyme or RNA were omitted were used as negative controls. Real-time PCR was performed with an ABI prism 7000 sequence detection system and SYBR green I dye reagents. The specific primers were designed by using the Primer 3 software. The following primers were used for real-time PCR: hGAPDH-RT-FP, 5'-TGGGCTACACTGAGCACCAG-3'; hGAPDH-RT-RP, 5'-GGGTGTCGCTGTTGAAGTCA-3'; hFasL-RT-FP, 5'-GGCCTGTGTCTCCTTGAT-3'; hFasL-RT-RP, 5'-TGCCAGCTCCTTCTGTAGGT-3'; hFas-RT-FP, 5'-TTGCTAGATTATCGTCCAAAAGTGTT-3'; hFas-RT-RP, 5'-AACAGTCTTCCTCAATTCATCC-3'. The pre-designed probes for human c-IAP-1, c-IAP-2 and GAPDH were purchased from Applied Biosystems with the following accession numbers: c-IAP-1, Hs00220419_m1; c-IAP-2, Hs00357350_m1; GAPDH, Hs00266705_q1.

The parameter C_t (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expressions of FasL, Fas and c-IAP-1/2 were analysed using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) by normalizing with GAPDH gene expression in all the experiments.

Reagents and antibodies. Anti-Human FasL antibody NOK-2 and the isotype IgG_{2a} control as well as caspase-8 inhibitor (Z-IETD-FMK) were purchased from BD Biosciences Pharmingen (Gregoli & Bondurant, 1999). β -Actin antibodies were purchased from Calbiochem. Helenalin (NF κ B-specific inhibitor) and p65 antibody were purchased from Biomol Research Laboratories. TLR2, caspase-3 and caspase-8 antibodies were purchased from Santa Cruz Biotechnology Inc. Phospho-NF κ B p65 antibody was purchased from Cell Signalling Technology.

FasL neutralization assay. FasL-dependent cell death was evaluated by a neutralization assay. Briefly, HGEC were treated with heat-inactivated *P. gingivalis* (5×10^7) and 1 h after incubation the NOK-2 antibody (50 µg ml⁻¹) or the isotype control (50 µg ml⁻¹) was added to the cells and then harvested after 24 h for DNA fragmentation assessment using the Cell Death ELISA kit (Roche).

Caspase-8 neutralization assay. Caspase-8-mediated cell death was evaluated with a neutralization assay. HGEC were pretreated with a caspase-8 inhibitor (10 mM) for 30 min and *P. gingivalis* (5×10^7) was added to the cultured cells. A DNA fragmentation assay was performed after 24 h.

NF κ B neutralization assay. The role of NF κ B in the apoptotic death of HGEC was analysed by the use of helenalin. Helenalin inhibits NF κ B by specific and irreversible alkylation of the p65 subunit, thereby blocking DNA binding (Lyss *et al.*, 1998). One hour prior to *P. gingivalis* treatment, 10 µM and 25 µM helenalin was added to the HGEC. Measurement of FasL mRNA by real-time PCR was performed.

DNA fragmentation ELISA assay. Treated HGEC cells were lysed after 24 h to measure apoptosis. DNA fragmentation was quantified using the Cell Death ELISA kit (Roche). The assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allows for the determination of mono- and oligonucleosomes present in the cytoplasmic fraction of cell lysates. The sample was placed into a streptavidin-coated microtitre plate. A mixture of antihistone-biotin-labelled antibody and anti-DNA peroxidase-conjugated antibody was added and incubated for 2 h. After removal of the antibodies by a washing step, the amount of nucleosome-bound fragmented DNA was quantified by the peroxidase retained in the immunocomplex using ABTS [9,2,2'-azino-di(3-ethylbenzthiazolin-sulfonate)] as a substrate.

Caspase activity assay. To measure caspase-8 activity, cytoplasmic extracts were prepared from HGEC cells treated for 24 h, and analysed using the caspase-8 activity assay kit (Chemicon International) as directed by the manufacturer.

Western blot analysis. Following treatment, cells were collected and lysed using DIGNAM buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 µg ml⁻¹ each leupeptin, pepstatin, leucine thiol, 0.1% Nonidet P-40). Cytoplasmic protein was extracted and the concentration determined using a protein assay reagent (Bio-Rad). Equal amounts (60 µg) of extracted proteins were resolved by 10% SDS-PAGE and subjected to standard immunoblotting procedures. The primary antibodies used were FasL (C-178) (1:500 dilution), caspase-8 (1:100), caspase-3 (1:100), TLR2 (1:200), phospho-p65 (1:200), c-IAP-1/2 (1:500), p65 (1:500) and β -actin (1:1000) purchased from Santa Cruz Biotechnology, except phospho-p65 antibody, which was purchased from Cell Signalling Technology, and p65, which was purchased from Biomol Research Laboratories. The appropriate secondary antibodies were used at a 1:2000 dilution. Protein signals were visualized using the ECL system (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The molecular sizes of the developed proteins were estimated by comparison with pre-stained protein markers (Invitrogen).

Transient transfection. Transient transfections were performed with 0.5×10^6 cells in six-well plates. Cells were transfected with 1 µg plasmid NF κ B-LUC (Stratagene) and 1 ng β -galactosidase as an internal control to monitor transfection efficiency, using Fugene 6 (Roche Diagnostics) for 24 h in MCDB 153 medium. Cells were treated with *P. gingivalis* (5×10^7 and 10×10^7). Cell extracts were collected after 8 h. Luciferase and β -galactosidase activities were measured using Promega kits according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA). Untreated and *P. gingivalis* cells treated for 30 min and 1 h were lysed in 10 mM HEPES 1⁻, pH 7.8, 1.5 mM MgCl₂ 1⁻, 0.5 mM DTT 1⁻, 0.1% Nonidet P-40, 0.5 µmol PMSF 1⁻ and 1 µmol 1⁻ each of the protease inhibitors aprotinin, leupeptin and leucine thiol. Nuclei were harvested and lysed in 20 mM HEPES 1⁻, pH 7.8, 520 mM NaCl 1⁻, 1.5 mM MgCl₂ 1⁻, 0.1 mM EDTA 1⁻, 25% glycerol, 0.5 mM DTT 1⁻ and 0.1% Nonidet P-40, with protease inhibitors. Samples were centrifuged at 100 000 g for 15 min and nuclear protein stored at -70 °C. EMSA was performed using double standard oligonucleotides containing the binding sites for

NF κ B (5'-AGTTGAGGGGACTTCCAGGC-3'; Promega) according to the manufacturer's instructions. The specificity of the reaction was evaluated using excess of unlabelled oligonucleotide.

Statistical analysis. All data are expressed as the mean $\pm$ SEM. Statistical analyses were performed by a Student's *t*-test. LDH activity assay was analysed by ANOVA and Tukey–Kramer multiple comparison test. A *P* value of <0.05 was considered significant.

RESULTS

P. gingivalis induces apoptotic cell death in HGEC

Exposure of HGEC to heat-killed *P. gingivalis* (5×10^7) suspended in MCDB 153 medium caused increased cell death and decreased viability as assessed by trypan blue dye exclusion staining [*P. gingivalis* 8 h, $72.3 \pm 10.5\%$, $P < 0.01$ versus untreated control (100 %); *P. gingivalis* 24 h, $40.23 \pm 6.7\%$, $P < 0.01$ versus untreated control (100 %)] (Fig. 1a). This was further supported by increased propidium iodide staining as measured by flow cytometry over a similar time frame (Fig. 1b, $P < 0.05$).

To determine whether the cell death was due to apoptosis the following studies were performed. First, the level of LDH in culture supernatants of HGEC after *P. gingivalis* treatment was examined. The LDH activity in total cells was measured after solubilization of cells with 0.1 % (v/v) Triton X-100 in total cells after removal of medium and washing the cells with PBS. There were no statistically significant differences at 8 and 24 h after *P. gingivalis* exposure in the levels of LDH in *P. gingivalis*-treated cells in comparison to untreated controls, indicating that death was not predominantly related to necrotic cell death (Fig. 1c).

In contrast, heat-killed *P. gingivalis* induced increased DNA fragmentation within 8 and 24 h, at which time a more than twofold increase was observed in comparison to untreated cells (Fig. 1d).

P. gingivalis up-regulates Fas, FasL and TLR2 expression in HGEC exposed to heat-killed *P. gingivalis*

In order to determine the mechanism of HGEC-mediated cell death by apoptosis after stimulation with *P. gingivalis*,

Fig. 1. Cell viability of HGEC after exposure to *P. gingivalis*. (a) Cell viability of HGEC after exposure to *P. gingivalis* determined by trypan blue exclusion. Results represent percentage of viable HGEC after exposure to *P. gingivalis* after 8 h and 24 h as determined by percentage of viability relative to untreated cells (UT=100 %). Values represent mean of triplicate samples. * $P < 0.01$ vs UT. (b) Propidium iodide staining of subconfluent HGEC before and after treatment with *P. gingivalis* as described in Methods. The survival of cultures is indicated for each condition. The experiments were repeated three times and data expressed as mean $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. (c) Amount of LDH release under each condition. Values are data from three independent experiments. Method of analysis used was ANOVA and Tukey–Kramer multiple comparison test. No significant difference in LDH activity was observed between the groups. (d) Induction of apoptosis by *P. gingivalis*. Level of histone-associated DNA fragments in HGEC was measured by a cell death ELISA kit. HGEC were untreated (UT) or incubated with *P. gingivalis* for 8 and 24 h. Data for the *P. gingivalis* samples were normalized to the untreated control and represent mean $\pm$ SEM of two assays. * $P < 0.05$.

we first examined the mRNA levels of Fas, FasL and TLR2 by RT-PCR and real-time PCR. A significant increase of FasL mRNA levels was detected by real-time PCR (*P. gingivalis* 8 h, 2.2 ± 0.3 times versus untreated control; *P. gingivalis* 24 h, 2.35 ± 0.5 times versus untreated control) (Fig. 2a). Furthermore, *P. gingivalis* induced increased FasL protein in HGEC as defined by Western blotting and detection of a 38 kDa band (Fig. 2c). A significant increase in TLR2 mRNA was also detected in HGEC stimulated with heat-killed *P. gingivalis* (Fig. 2b). HGEC constitutively expressed TLR2 but at low levels. After 8 h *P. gingivalis* stimulation, TLR2 mRNA was strongly increased. However, TLR2 mRNA levels were noted to decrease after 24 h stimulation with *P. gingivalis*. These changes in TLR2 levels were not confirmed by Western blot analysis, which revealed a stable level of TLR2 protein expression after *P. gingivalis* treatment, perhaps reflecting a long half-life of the stimulated protein (Fig. 2c). An up-regulation of TLR2 was also detected in HGEC after exposure to *P. gingivalis* LPS (P-LPS) and outer-membrane protein (OMP) extracted from *P. gingivalis* (data not shown). The level of TLR4 mRNA was observed to be very low, with no detectable up-regulation after bacterial stimulation (data not shown). Finally, we examined Fas expression in HGEC exposed to *P. gingivalis*. These studies

revealed a significant and sustained up-regulation of Fas mRNA levels by 8 h stimulation (Fig. 2d).

***P. gingivalis* up-regulates FasL expression and activates caspase-8 and caspase-3**

To investigate the effect of up-regulated FasL expression induced by *P. gingivalis*, molecules downstream of FasL were examined. Treatment of HGEC with *P. gingivalis* for 8 h and 24 h led to a reduction of procaspase-8 (55/50 kDa) levels, with a corollary accumulation of the cleavage product procaspase-8 (40/36 kDa; Fig. 3a). Similar results were observed for caspase-3, wherein the latent form (p32) was observed to be cleaved into the catalytically active effector protease (p17) (Fig. 3b).

To examine the functionality of caspase-8 induced by *P. gingivalis*, we performed a caspase-8 activity assay (Fig. 3c). These studies showed that *P. gingivalis* induced significant activation of caspase-8 [75 ± 6.9 units (mg protein) $^{-1}$ versus 8.9 ± 0.8 units (mg protein) $^{-1}$]. Interestingly, the anti-apoptotic survival proteins c-IAP-1/c-IAP-2 were observed to decrease in HGEC stimulated with *P. gingivalis* after 8 and 24 h as assessed by real-time PCR (Fig. 3d, e) and Western blot analysis (Fig. 3f). Together, these studies are

Fig. 2. Effect of *P. gingivalis* on FasL and TLR2 mRNA expression in HGEC. (a) Induction of FasL mRNA expression after exposure to *P. gingivalis* (5×10^7) after 8 h and 24 h determined by real-time PCR. Error bars represent SEM of duplicate samples; $*P < 0.05$. TLR2 mRNA is induced after stimulation with *P. gingivalis*. (b) Total RNA was isolated at 8 h and 24 h from HGEC untreated (UT) or stimulated by *P. gingivalis* (5×10^7) and subjected to RT-PCR analysis for the presence of mRNAs encoded by TLR2 (658 bp). β -Actin expression was used as a normalizing control. (c) Western blot analysis of TLR2 and FasL. Forty micrograms of total protein from cell extracts of untreated HGEC and *P. gingivalis*-treated HGEC after 8 h and 24 h were resolved by SDS-PAGE and assayed for TLR2 and FasL by using respective antibodies as described in Methods. (d) Induction of Fas mRNA expression after exposure to *P. gingivalis* (5×10^7) after 8 h and 24 h determined by real-time PCR. Error bars represent SEM of duplicate samples; $*P < 0.05$.

Fig. 3. Western blot analysis of caspase-8 and caspase-3. Activation of caspase-8 and -3 in HGEC stimulated with *P. gingivalis* as shown by the cleavage of caspase-8 (a) and caspase-3 (b) as detected by their specific antibodies. Detection of β -actin was used to verify equal concentrations of protein. (c) Activation of caspase-8 in HGEC exposed to *P. gingivalis*. Caspase-8 activity was measured using a caspase-8 Colorimetric Activity Assay kit. Data are presented as mean $\pm$ SEM of two assays (* $P < 0.05$). (d, e) Decreased c-IAP-1 (d) and c-IAP-2 (e) mRNA levels were detected in HGEC stimulated with heat-killed *P. gingivalis*. (f) Western blot analysis of c-IAP-1/2 protein expression in untreated and *P. gingivalis*-treated HGEC. Detection of β -actin was used to verify equal concentrations of protein.

consistent with induction of an epithelial phenotype that is pro-apoptotic in HGEC after *P. gingivalis* stimulation.

Requirement for Fas–FasL interactions in *P. gingivalis*-induced apoptosis in HGEC

To confirm that the *P. gingivalis*-induced apoptosis of HGEC was mediated through a Fas/FasL signalling pathway, the following experiments were performed. HGEC were pre-treated with the NOK-2 (anti-FasL) antibody (50 $\mu\text{g ml}^{-1}$) to block Fas–FasL interactions before exposure to heat-killed *P. gingivalis* (Fig. 4a). NOK-2 pretreatment completely abrogated apoptotic HGEC cell death induced by *P. gingivalis* as assessed by a cytoplasmic histone-associated DNA fragmentation assay. IgG control antibody alone did not induce apoptosis whereas IgG control with *P. gingivalis* induced DNA fragmentation as observed in Fig. 4(a). In addition, HGEC were treated with a caspase-8 inhibitor (Z-IETD-FMK, 10 mM) and apoptosis assessed by a DNA fragmentation ELISA assay. The caspase-8-specific inhibitor blocked DNA fragmentation induced by *P. gingivalis* (Fig. 4b). These data suggest that FasL-mediated, caspase-8-dependent apoptotic cell death caused by up-regulation of

FasL expression is the major cell death pathway in HGEC exposed to heat-killed *P. gingivalis*.

Activation of NF κ B-induced FasL expression in HGEC stimulated with heat-killed *P. gingivalis*

It is well known that NF κ B is an important regulator of FasL expression (Farhana *et al.*, 2005). We therefore sought to investigate NF κ B activation in HGEC stimulated by heat-killed *P. gingivalis* by transient transfection of HGEC with an NF κ B luciferase vector. These luciferase studies showed that *P. gingivalis* stimulation of HGEC activated the NF κ B promoter (Fig. 5a). We next examined NF κ B activation by assessment of phosphorylated (p) p65 levels. It was observed that unstimulated HGEC exhibited no evidence of pp65. However, exposure of HGEC to heat-killed *P. gingivalis* induced a strong increase in pp65 (Fig. 5b). Activation of NF κ B was studied in nuclear extracts from untreated cells, and cells stimulated with *P. gingivalis* for 30 min and 1 h. Increased DNA-binding activity of NF κ B by EMSA was detected (Fig. 5c).

To confirm that NF κ B regulated FasL expression in HGEC stimulated by *P. gingivalis*, we performed the following

Fig. 4. Induction of apoptosis by *P. gingivalis* and abrogation of apoptotic cell death by anti-FasL antibody and caspase-8 inhibitor in HGEc. (a) Induction of apoptosis by *P. gingivalis* and abrogation of *P. gingivalis*-induced apoptosis by anti-FasL (NOK-2) antibody. Detection of histone-associated DNA fragments in HGEc was measured by a cell death ELISA kit. HGEc were untreated (UT) or incubated with *P. gingivalis* for 24 h, NOK-2 treated 1 h after *P. gingivalis* exposure, control IgG_{2a} treated 1 h after *P. gingivalis* exposure and IgG_{2a} control. Data are normalized to the untreated control and represent mean $\pm$ SEM of two assays. * $P < 0.001$. (b) Inhibition of apoptosis in HGEc stimulated with *P. gingivalis* by caspase-8 inhibitor Z-IETD-FMK. HGEc were untreated, treated with caspase-8 inhibitor Z-IETD-FMK (100 μ M) 30 min prior to *P. gingivalis* exposure or treated with caspase-8 inhibitor alone. Cytoplasmic extracts were prepared and analysed for DNA fragmentation using the Cell Death ELISA kit. Data are normalized to the untreated control and represent mean $\pm$ SEM of two assays.

study. Addition of an irreversible inhibitor of NF κ B activation, helenalin (1 μ M and 5 μ M), blocked FasL mRNA induction in HGEc stimulated with heat-killed *P. gingivalis* (Fig. 5d; * $P < 0.01$). In contrast, a caspase-8 inhibitor had no effect on FasL mRNA levels in HGEc stimulated with heat-killed *P. gingivalis*.

DISCUSSION

Apoptosis, or programmed cell death, is a fundamental biological process that is required by multicellular organisms to eliminate redundant, damaged or infected cells and, as such, maintain normal development, integrity and

homeostasis. The Fas (Apo-1/CD95)/FasL system is one of the key regulators of apoptosis in mammalian cells (Hsu *et al.*, 1999). Cross-linking of Fas by its natural ligand, FasL, or agonistic antibodies, can induce apoptosis in Fas-expressing cells (Chinnaiyan *et al.*, 1995, 1996). The crucial role of FasL in the regulation of immune homeostasis strongly indicates that its expression must be tightly regulated. Identification of the pathways regulating FasL expression is highly important in understanding the function and survival of oral gingival epithelial cells and consequently immune responses in periodontal tissues.

The early events in the FasL-initiated signalling pathway have been well characterized. Triggering of Fas by FasL leads to Fas-trimerization. Following Fas-FasL ligation, the recruitment of the serine-phosphorylated adapter FADD occurs. Recruitment of FADD and procaspase-8 leads to formation of a DISC. After procaspase-8 is recruited to the DISC, it undergoes its proteolytic activation to caspase-8, followed by initiation of a caspases cascade that leads to apoptotic cell death.

Recent work has shown that apoptosis is induced in periodontal tissues by host and microbial factors and may play an important role in the regulation of mucosal inflammation (Gamonal *et al.*, 2001; Sugiyama *et al.*, 1996; Tonetti *et al.*, 1994, 1998). It has been previously established that FasL, Fas, caspase-3 and p53 can be detected in the inflammatory infiltrates of biopsies obtained from periodontitis patients. Most of the TUNEL-positive cells detected were observed to be neutrophils in the inflammatory infiltrates and epithelial cells (Gamonal *et al.*, 2001; Tonetti *et al.*, 1998). However, Koulouri *et al.* (1999) found fibroblasts to be predominant apoptotic cell type in adult periodontitis lesions. Studies have shown that *P. gingivalis*, a Gram-negative anaerobic bacterium, is strongly associated with the onset of adult periodontitis (Nakhjiri *et al.*, 2001; Offenbacher, 1996; Savit & Socransky, 1984; Sugiyama *et al.*, 1996). However, not all individuals infected with this bacterium are equally susceptible to bone resorption, suggesting that the host response is important in the onset and progression of the disease (Savit & Socransky, 1984). *P. gingivalis* contains a vast array of virulence factors, toxic metabolites and cellular constituents that interact with cell surface receptors on epithelial cells, such as gingipain proteinases, OMP, butyric acid, fimbriae and LPS (Nakhjiri *et al.*, 2001; Sandros *et al.*, 2000). Many studies have shown that *P. gingivalis* challenge of epithelial cells can elicit cytokine responses (Sandros *et al.*, 2000). Nakhjiri *et al.* (2001) observed inhibition of apoptosis of epithelial cells after stimulation with *P. gingivalis*. In our study, heat-killed *P. gingivalis* induced apoptotic cell death of HGEc through up-regulation of FasL and activation of the caspases cascade. OMP extracted from *P. gingivalis* induced the same response of HGEc as the heat-killed bacteria regarding FasL up-regulation and DNA fragmentation (data not shown) in our study. Knowledge of the mechanisms of stimulation and response of HGEc by *P. gingivalis* needs to be elucidated in order to understand

Fig. 5. Activation of NF κ B-induced FasL expression in HGEC exposed to *P. gingivalis*. (a) Transient transfection of HGEC with NF κ B luciferase reporter gene and β -galactosidase construct (for normalization of transfection efficiency) by Fugene. Luciferase and β -galactosidase assays were performed following treatment of cells in the absence or presence of *P. gingivalis*. * P <0.01. (b) Western blotting with phospho-NF κ B p65 (pp65) antibody. The same samples were immunoblotted with β -actin antibodies to assess protein loading. (c) DNA-binding activity of NF κ B by EMSA. Activation of NF κ B was studied in nuclear extracts from untreated cells (UT) and cells treated for 30 min and 1 h with *P. gingivalis*, respectively. (d) Suppression of FasL mRNA by NOK-2 and helenalin in HGEC exposed to *P. gingivalis*. The levels of FasL and the internal control (GAPDH) were analysed by quantitative real-time PCR as described in Methods. Inhibition of FasL mRNA by NOK-2 and helenalin was observed and expressed as fold change over UT. * P <0.01. A caspase-8 inhibitor did not suppress FasL mRNA expression by *P. gingivalis*.

the pathogenicity of *P. gingivalis* in periodontal tissue. Further studies are needed to clarify pro- and anti-apoptotic properties of *P. gingivalis* in HGEC.

In particular, the recognition and transduction of those bacterial signals involves the transmembrane TLRs. It has been previously suggested that TLR2 is activated by *P. gingivalis* (Yoshimura *et al.*, 2002). Although early studies suggested that this TLR2 activation is due to LPS from *P. gingivalis* (Yoshimura *et al.*, 2002), more recent studies have shown that TLR2 recognizes unknown cell wall components rather than LPS itself in *P. gingivalis* interactions (Yoshimura *et al.*, 2003). HGEC exposure to *P. gingivalis* increased mRNA and protein levels of TLR2, but not TLR4, and the synthesis and release of an array of proinflammatory cytokines including TNF- α , IL-1, IL-6, IL-8, IL-18 (data not shown) and up-regulation of FasL. Sandros *et al.* (2000) observed strong cytokine response in both KB cells and primary cultures after stimulation with *P. gingivalis*. Similarly, Kasumoto *et al.* (2004) showed strong mRNA expression of TLR2, TLR4, TLR5 and TLR9 in HGEC stimulated with sonic extracts of *P. gingivalis*. They showed that TLR2 participates in the signalling pathway to induce chemokines in an

NF κ B-dependent pathway. They also observed differences in expression of TLRs in different epithelial cell lines.

NF κ B is known to regulate this constellation of cytokines. NF κ B is an essential transcription factor in the control of expression of genes involved in immune and inflammatory response. It has been previously shown that TNF- α through phosphorylation of Rel/p65 increases transcriptional activity but does not affect nuclear translocation or DNA-binding activity (D. Wang *et al.*, 1998). NF κ B activation serves as a primary mechanism to protect cells against apoptotic stimulus such as TNF, most likely upon activation of TRAF1 and TRAF2, c-IAP-1 and c-IAP-2 (C.-Y. Wang *et al.*, 1998).

However, our results showed that *P. gingivalis* did stimulate HGEC to increase expression of TLR2, Fas and FasL in a sequential fashion. Moreover, the up-regulation of FasL was dependent on NF κ B pathways. In contrast to the findings of C.-Y. Wang *et al.* (1998), our study did not detect activation of c-IAP-1/2 and suppression of apoptosis upon activation of NF κ B. Yilmaz *et al.* (2004) showed that *P. gingivalis* survival in primary epithelial cells is mediated through activation of the phosphatidylinositol 3-kinase/Akt pathway

which down-regulates the inflammatory response and promotes cell survival. However, in concordance with our data, Farhana *et al.* (2005) showed that the pro-apoptotic effect of NF κ B activation after stimulation by retinoid-related molecule (3-Cl-AHPC) is through inhibition of anti-apoptotic proteins c-IAP-1, XIAP and Bcl-X_L and enhanced expression of pro-apoptotic molecules in a number of cell types. Thus, it appears that NF κ B activation is likely to be both anti-apoptotic and pro-apoptotic, depending on the stimulus and the specific cell type involved. Alikhani *et al.* (2005) showed the major role of FOXO1 in controlling apoptosis. Based on their report, it would be important to investigate the role of FOXO1 in *P. gingivalis*-stimulated HGEC.

The increase in FasL expression observed was accompanied by caspase-8 activation and DNA fragmentation, leading to apoptotic cell death. Additionally, apoptosis induced by *P. gingivalis* in HGEC was blocked by incubation with a neutralizing FasL antibody, demonstrating the functional relevance of increased FasL expression under these conditions. After incubation of HGEC with a caspase-8 inhibitor, apoptosis was also blocked. This further indicates that FasL-mediated apoptosis of HGEC exposed to *P. gingivalis* occurs via a cytoplasmic pathway involving activation of caspases. Since cytosolic procaspase-8 has to be recruited to the Fas-associated DISC to become activated, our data imply that heat-killed *P. gingivalis* leads to the formation of the DISC and induction of caspase-8-dependent apoptotic cell death in HGEC.

In summary, we show that NF κ B activation is required for FasL-induced apoptosis in HGEC during *P. gingivalis* infection. These studies reveal an important pathophysiological relationship between *P. gingivalis* and primary gingival epithelial cells in humans. Although it remains to be determined how these events affect clinical disease, our findings predict that blockade of *P. gingivalis* interaction may be of potential therapeutic benefit.

ACKNOWLEDGEMENTS

R. S. Blumberg was supported by NIH grants DK44319, DK51362 and DK53056. D. F. Kinane was supported by grants HRSA-C76HF01199-01, CDC-PA04189 and CDC H7S/CCU424835-01.

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