Porphyromonas gingivalis enhances FasL expression via up-regulation of NFκB-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-κB (NFκB) 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 NFκB by P. gingivalis in HGEC was demonstrated by an NFκB 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 NFκB 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.

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 periodontopathogenic 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)-1β, tumour necrosis factor (TNF)-z, 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...
signalling, which trigger an intracellular cascade that leads to activation of the transcription factor nuclear factor-κB (NFκB). 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 periodontal disease. P. gingivalis by bacterial pathogens is important in many infectious diseases. TLR1, TLR2 and TLR6 have shown expression of TLR1, TLR2 and TLR6. However, 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-1β-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 NFκB gene transcription and activation of NFκB. 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 triplicate 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 μg insulin ml⁻¹, 5 μg transferrin ml⁻¹, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 mM sodium selenite supplemented with 50 μg bovine pituitary extract ml⁻¹, 100 units penicillin ml⁻¹, 100 μg 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 x 10⁶ 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 x 10⁷), 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.
NFκB neutralization assay. The role of NFκB in the apoptotic death of HGEC was analysed by the use of helenalin. Helenalin inhibits NFκB 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-6-sulfonate)] as a substrate.

Caspace 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. Both *P. gingivalis* (5×10⁶ and 10×10⁶) 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 mmol HEPES 1/10, pH 7.8, 1.5 mmol MgCl₂ 1/3, 0.5 mmol DTT 1/1, 0.1% Nonidet P-40, 0.5 μmol PMSF 1/1 and 1 μmol 1/1 each of the protease inhibitors aprotinin, leupeptin and leucine thiol. Nuclei were harvested and lysed in 20 mmol HEPES 1/1, pH 7.8, 520 mmol NaCl 1/3, 1.5 mmol MgCl₂ 1/3, 0.1 mmol EDTA 1/1, 25% glycerol, 0.5 mmol DTT 1/1 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 NFκB.
NFkB (5'-AGTTGAGGGACTTTCCCAGGC-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 ± 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 × 10⁷) 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 ± 10.5 %, P<0.01 versus untreated control (100 %); *P. gingivalis* 24 h, 40.23 ± 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*,...
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 \((P.\ gingivalis\ 8\ h, 2.2 \pm 0.3\ times\ versus\ untreated\ control;\ P.\ gingivalis\ 24\ h, 2.35 \pm 0.5\ times\ versus\ untreated\ control)\) (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 \(\left[75 \pm 6.9\ \text{units}\ \left(\mathrm{mg}\ \text{protein}\right)^{-1}\right]\) versus \(8.9 \pm 0.8\ \text{units}\ \left(\mathrm{mg}\ \text{protein}\right)^{-1}\). 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 significant and warrants further investigation.

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**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 \(\times\) \(10^7\)) 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 \(\times\) \(10^7\)) and subjected to RT-PCR analysis for the presence of mRNAs encoded by TLR2 (658 bp). \(\beta\)-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 \(\times\) \(10^7\)) after 8 h and 24 h determined by real-time PCR. Error bars represent SEM of duplicate samples; \(*P < 0.05\).
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 μg 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 NFκB-induced FasL expression in HGEC stimulated with heat-killed *P. gingivalis***

It is well known that NFκB is an important regulator of FasL expression (Farhana et al., 2005). We therefore sought to investigate NFκB activation in HGEC stimulated by heat-killed *P. gingivalis* by transient transfection of HGEC with an NFκB luciferase vector. These luciferase studies showed that *P. gingivalis* stimulation of HGEC activated the NFκB promoter (Fig. 5a). We next examined NFκB 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 NFκB 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 NFκB by EMSA was detected (Fig. 5c).

To confirm that NFκB regulated FasL expression in HGEC stimulated by *P. gingivalis*, we performed the following
P. gingivalis induces FasL and apoptosis

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 ± SEM of two assays. *P<0.01. (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 μM) 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 ± SEM of two assays.

study. Addition of an irreversible inhibitor of NFκB activation, helenalin (1 μM and 5 μM), 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
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 NFκB-dependent pathway. They also observed differences in expression of TLRs in different epithelial cell lines.

NFκB is known to regulate this constellation of cytokines. NFκB 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). NFκB 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 NFκB 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 NFκB. Yilmaz et al. (2004) showed that P. gingivalis survival in primary epithelial cells is mediated through activation of the phosphatidylinositol 3-kinase/Akt pathway.

**Fig. 5.** Activation of NFκB-induced FasL expression in HGEC exposed to P. gingivalis. (a) Transient transfection of HGEC with NFκB 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-NFκB p65 (pp65) antibody. The same samples were immunoblotted with β-actin antibodies to assess protein loading. (c) DNA-binding activity of NFκB by EMSA. Activation of NFκB 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.
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 NFκB activation after stimulation by retinoid-related molecule (3-Cl-AHPC) is through inhibition of anti-apoptotic proteins c-IAP-1, XIAP and Bcl-2, and enhanced expression of pro-apoptotic molecules in a number of cell types. Thus, it appears that NFκB 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 HGE.

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 HGE 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 caspasas. 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 HGE.

In summary, we show that NFκB activation is required for FasL-induced apoptosis in HGE 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.

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