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Fibroblast apoptosis induced by *Porphyromonas gingivalis* is stimulated by a gingipain and caspase-independent pathway that involves apoptosis-inducing factor

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Summary

Porphyromonas gingivalis is an oral bacterium that causes pathology in a number of dental infections that are associated with increased fibroblast cell death. Studies presented here demonstrated that *P. gingivalis* stimulates cell death by apoptosis rather than necrosis. Unlike previous studies apoptosis was induced independent of proteolytic activity and was also independent of caspase activity because a pan-caspase inhibitor, Z-VAD-fmk, had little effect. Moreover, *P. gingivalis* downregulated caspase-3 mRNA levels and caspase-3 activity. The consequence of this downregulation was a significant reduction in tumour necrosis factor-α-induced apoptosis, which is caspase-3-dependent. Immunofluorescence and immunoblot analysis revealed *P. gingivalis*-induced translocation of apoptosis-inducing factor (AIF) from the cytoplasm to the nucleus. siRNA studies were undertaken and demonstrated that *P. gingivalis* stimulated cell death was significantly reduced when AIF was silenced ($P < 0.05$). Treatment of human gingival fibroblasts with H-89, a protein kinase A inhibitor that blocks AIF activation also reduced *P. gingivalis*-induced apoptosis ($P < 0.05$). These results indicate that *P. gingivalis* causes fibroblast apoptosis through a pathway that involves protein kinase A and AIF, is not dependent upon bacterial proteolytic activity and is also independent of the classic apoptotic pathways involving caspase-3.

Introduction

Porphyromonas gingivalis is a Gram-negative anaerobic organism that is usually absent or detected at low levels in health but is frequently found at elevated levels in the periodontal pockets of patients with periodontitis, an inflammatory disease of tooth-supporting tissues (Lamont and Jenkinson, 1998; Graves *et al*., 2001). *P. gingivalis* produces a variety of virulence factors that affect the host response including lipopolysaccharide (LPS), fimbriae and proteases (Graves *et al*., 2000). These virulence factors may promote colonization, stimulate inflammation, or enhance survival of *P. gingivalis* through direct and indirect mechanisms (O'Brien-Simpson *et al*., 2005; Takii *et al*., 2005). Much attention has recently been focused on *P. gingivalis* proteases, most importantly, gingipains, which degrade proteins at arginine or lysine residues. These proteases may enable the bacterium to evade the host response by degrading host proteins, enhance invasion and colonization and provide nutritional support (Sheets *et al*., 2005). Furthermore, gingipain proteases may affect the host by inducing apoptosis of cells (Wang *et al*., 1999).

Emerging evidence indicates that bacteria-induced apoptosis is an important phenomenon in several pathologic conditions. For example, *H. pylori* contributes to the development of gastric

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ulcers by inducing apoptosis of gastric epithelial cells (Basak *et al*., 2005). Similarly, the morbidity of bacterial meningitis is aggravated by bacteria-induced neuronal cell apoptosis (Braun *et al*., 2001). In the periodontium the loss of fibroblasts is a characteristic features associated with progressive periodontal disease (Zappa *et al*., 1992). Apoptosis of fibroblasts is associated with inflammation in human gingiva (Tonetti *et al*., 1998; Koulouri *et al*., 1999). Moreover, *P. gingivalis*-induced fibroblast apoptosis is mediated to a large extent by tumour necrosis factor (TNF) and is aggravated by conditions such as diabetes, which is associated with more severe periodontal disease (Graves *et al*., 2001; Graves *et al*., 2006; Liu *et al*., 2006). However, there is evidence that *P. gingivalis* can directly induce apoptosis through bacterial cell wall components, particularly gingipain proteases (Kurita-Ochiai *et al*., 1998; Imamura *et al*., 2003). Gingipain proteases from bacterial culture supernatant have been reported to cause cell rounding and death by apoptosis (Wang *et al*., 1999; Chen *et al*., 2001; Sheets *et al*., 2005). In contrast, live *P. gingivalis* has been reported to be antiapoptotic for epithelial cells (Nakhjiri *et al*., 2001) while heat killed *P. gingivalis* is pro-apoptotic (Brozovic *et al*., 2006).

Two widely recognized mechanisms by which apoptosis occur involve the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is triggered by death activators (ligands) that bind to receptors at the cell surface. Death promoting signals are frequently associated with molecules termed death activators, such as cytokines belonging to the TNF family, namely TNF-α, lymphotoxin, FasL (fas ligand), Apo3L and TRAIL (TNF-related apoptosis-inducing ligand) (Thorburn, 2004; Thomas *et al*., 2004). The intrinsic pathway is generated by signals arising from within the cell in response to cell damage. For example, it is induced by conditions that cause cell damage such as exposure to *reactive oxygen species*, ionizing radiation or chemotherapeutic agents (Sordet *et al*., 2004). A *third* less well-understood mechanism involves molecules such as apoptosis-inducing factor (AIF). AIF is a protein that is normally located in the intermembrane space of mitochondria. When the cell receives signals for death, AIF is released from the mitochondria, similar to the release of cytochrome *c* in the *intrinsic* pathway and translocates to the nucleus where it facilitates DNA fragmentation. Interestingly, AIF does not require caspases to induce apoptosis (Cande *et al*., 2002; Lipton and Bossy-Wetzel, 2002). Based on previous reports we investigated mechanisms by which *P. gingivalis* induced apoptosis focusing initially on bacterial proteases and caspase-mediated cell death since it had been reported that *P. gingivalis* protesases induce fibroblast apoptosis through caspase-3 (Sheets *et al*., 2005). Contrary to expectations *P. gingivalis*-induced apoptosis of human gingival fibroblasts was largely independent of proteolytic enzymes or caspase-3. Further studies demonstrated that *P. gingivalis* downregulated caspase-3 activity and as a result reduced TNF-α-induced apoptosis. *P. gingivalis*, however, induced activation of the AIF pathway as demonstrated by enhanced nuclear translocation of AIF and *P. gingivalis* stimulated apoptosis was significantly inhibited by AIF siRNA, and by H-89, a protein kinase A inhibitor that has been shown to block AIF activation.

Results

Time and dose–response experiments were undertaken to examine the impact of *P. gingivalis* on cell rounding and induction of apoptosis in human gingival fibroblasts. Morphological alterations and apoptosis of fibroblasts were examined after exposure to live *P. gingivalis* [multiplicity of infection (moi) 100:1, 300:1 and 900:1] for 3 h (Fig. 1). At the lowest concentration *P. gingivalis* had no effect on cell rounding but did induce apoptosis ($P < 0.05$). Both parameters were increased at higher concentrations. Cell rounding is consistent with the degradation of cell-extracellular matrix adhesion molecules by bacterial proteolytic enzymes (Chen *et al*., 2001;Sheets *et al*., 2005). Only small amounts of LDH were released into culture media for all doses tested indicating that cell death occurred via apoptosis but not necrosis ($P > 0.05$). A similar pattern was obtained when a time-course experiment was carried

out. At the initial time point, 1.5 h, *P. gingivalis* caused little cell rounding but did enhance apoptosis ($P < 0.05$). With longer time points there was an increase in cell rounding and apoptosis but only a very small increase in necrosis. Thus, temporal separation of *P. gingivalis*-induced cell rounding and apoptosis was noted at early time points and low concentrations of bacteria. To investigate a mechanism by which *P. gingivalis*-induced apoptosis protease inhibitors were used. Leupeptin (an Arg-gingipain inhibitor) abolished *P. gingivalis*-induced morphological rounding (Fig. 2A) consistent with published data (Johansson and Kalfas, 1998;Wang *et al*., 1999;Chen *et al*., 2001). Leupeptin did not significantly alter the level of apoptosis ($P > 0.05$) (Fig. 2B). When cells were incubated with leupeptin plus cathepsin B inhibitor-II, a lys-gingipain inhibitor (Houle *et al*., 2003) no further reduction in apoptosis occurred (data not shown). When apoptosis was assessed by the TUNEL assay proteolytic inhibitors did not significantly reduce apoptosis ($P > 0.05$) (Fig. 2C). Similar results were obtained with proteins released by *P. gingivalis* that have proteolytic activity and commercially available endonucleases. Both caused extensive cell rounding (data not shown) but neither induced significant levels of fibroblast apoptosis (Fig. 2D). Thus, proteolytic enzyme contributed little to *P. gingivalis*-induced apoptosis.

Apoptotic cell death typically occurs by stimulating caspases (Thorburn, 2004). Caspase-3 is the principle effector caspase of the well-defined cytosolic and mitochondrial pathways (Green, 2000). To examine the effect of *P. gingivalis* on caspase-3 expression real-time polymerase chain reaction (PCR) was carried out and compared with a positive control, TNF-α. TNF-α increased caspase-3 mRNA levels by 2.3-fold (Fig. 3A). In contrast, *P. gingivalis* did not enhance caspase-3 mRNA levels ($P > 0.05$). To determine whether *P. gingivalis* induced apoptosis through a caspase-dependent mechanism the pancaspase inhibitor, Z-VAD-fmk, was used. TNF-α stimulated apoptosis in fibroblasts was significantly inhibited by Z-VAD-fmk ($P < 0.05$) (Fig. 3B). In contrast, the pancaspase inhibitor had no effect on *P. gingivalis*-induced apoptosis ($P > 0.05$). We also examined the effect of a pan-caspase inhibitor on *P. gingivalis* (moi 100:1) stimulated apoptosis over a 24 h period and found that it did not reduce *P. gingivalis*-induced apoptosis (data not shown). Consistent to our result Urnowey and colleagues (2006) showed little to no increase in caspase-3 and -7 at 24 h of bacterial infection. The impact of *P. gingivalis* on caspase mediated apoptosis was investigated further. In a pilot study human gingival fibroblasts did not undergo apoptosis when exposed to *P. gingivalis* for 45 min followed by thorough rinsing (data not shown). To determine whether *P. gingivalis* modulated TNF-α-induced apoptosis cells were preincubated with *P. gingivalis* for 45 min, rinsed thoroughly and then incubated with TNF-α or a second incubation with *P. gingivalis*. Cells were assayed for caspase-3/7 activity or apoptosis. Pretreatment with *P. gingivalis* significantly inhibited TNF-α-induced caspase-3/7 activity (Fig. 4A). When apoptosis was measured, pretreatment of cells with *P. gingivalis* substantially reduced TNF-α-induced apoptosis but had no effect on the capacity of *P. gingivalis* itself to induce apoptosis (Fig. 4B). These results therefore indicate that *P. gingivalis* inhibits TNF-α-induced apoptosis by downregulating caspase-3 activity.

In order to investigate caspase-independent apoptosis experiments were performed focusing on AIF (Susin *et al*., 2000). When AIF is activated it translocates to the nucleus (Cande *et al*., 2002). Confocal microscopy of cells to assess AIF nuclear translocation was carried out using an anti-AIF antibody (Fig. 5A). The number of cells clearly demonstrating translocation of AIF to the nucleus increased threefold when compared with the unstimulated cells (Fig. 5B). siRNA studies were then carried out to functionally examine the role of AIF in *P. gingivalis*-induced apoptosis. AIF mRNA levels were reduced by approximately 90% by AIF siRNA compared with scrambled siRNA demonstrating effective silencing (Fig. 6A). This result was further confirmed using Western Blot analysis. AIF siRNA significantly reduced the presence of AIF in the cytoplasm as well as in the nucleus as compared with cells transfected with scrambled siRNA (Fig. 6B). In addition, *P. gingivalis* increased the level of AIF in the nuclear

compartment compared with unstimulated cells, agreeing with data obtained by confocal microscopy in Fig. 5B. When the effect on apoptosis was measured AIF silencing significantly reduced *P. gingivalis* stimulated apoptosis by almost half ($P < 0.05$) (Fig. 6C). Furthermore, a protein kinase A inhibitor (H-89) shown to inhibit AIF activation (Park *et al*., 2005) also reduced *P. gingivalis*-induced apoptosis by approximately 50% ($P < 0.05$) (Fig. 6D). H-89 is non-toxic and does not stimulate apoptosis of fibroblasts at the concentration used.

Discussion

The impact of *P. gingivalis* on cell death has been controversial. Several investigators have reported that it induces cell death through gingipain activity (Johansson and Kalfas, 1998; Wang *et al*., 1999; Sheets *et al*., 2005) while others reported that gingipain proteases do not play a significant role (Morioka *et al*., 1993; Harris *et al*., 2002). It has also been reported that *P. gingivalis* reduces apoptosis in epithelial cells (Nakhjiri *et al*., 2001). One possible explanation for the different findings is that in many of the reports gingipain-induced apoptosis was not compared with that induced by intact *P. gingivalis*. Studies presented here clarify a mechanism through which *P. gingivalis* induces apoptosis. While high concentrations of *P. gingivalis* stimulate necrosis *in vivo* (Liu *et al*., 2004) we found that the bacterium directly induced relatively little necrosis in fibroblasts *in vitro*. Thus, the principal mechanism by which *P. gingivalis* induced cell death was through apoptosis when an infective and not a toxic number of bacteria were incubated with the cells. Moreover, the pro-apoptotic effect of *P. gingivalis* was separated from its proteolytic activity in time-course and dose–response experiments and by findings that protease inhibitors blocked cell rounding while having only a small impact on apoptosis compared with viable *P. gingivalis*. This was established using both the ELISA assay for cytoplasmic histone-associated DNA and the TUNEL assay.

The studies establish for the first time that *P. gingivalis* does not induce fibroblast apoptosis through the well-characterized caspase-dependent apoptotic pathways. This is based on findings that *P. gingivalis*-induced apoptosis was not affected by a pan-caspase inhibitor, Z-VAD-fmk, while this inhibitor blocked apoptosis induced by TNF-α. Consistent with this observation, *P. gingivalis* downregulated caspase-3 activity. This is physiologically significant because pretreatment of cells with *P. gingivalis* followed by rinsing and incubation with TNF-α blocked TNF-α stimulated apoptosis. However, pretreatment with *P. gingivalis* did not inhibit apoptosis induced by a second incubation with *P. gingivalis* largely because it does not use a caspase-3-dependent pathway.

Although it has been proposed that *P. gingivalis* gingipains promote fibroblast apoptosis through activation of caspase-3 (Sheets *et al*., 2005) we found no evidence that *P. gingivalis* induced caspase-3 activity measured with a sensitive luminescent kit, but rather, downregulated it. In addition we examined the effect of a pan-caspase inhibitor on *P. gingivalis* stimulated apoptosis over a 24 h period and found that it did not reduce *P. gingivalis*-induced apoptosis (data not shown). In contrast, long-term exposure of cells to Pg-proteases will cause extensive cell detachment and promote apoptosis due to loss of attachment. Since incubation with *P. gingivalis* from 3 to 24 h induced apoptosis under conditions where caspase-3 activity was blocked, it is unlikely that indirect mechanisms through protease-induced detachment play a prominent role in fibroblast apoptosis stimulated by intact *P. gingivalis*. Urnowey and colleagues (2006) demonstrated that *P. gingivalis* at early infection- (< 6 h) induced expression of antiapoptotic proteins in fibroblasts. Similarly, Nakhjiri *et al.* (2001) showed an increase in expression of antiapoptotic protein bcl-2 when epithelial cells were treated with *P. gingivalis* for 24 h, which in turn reduced apoptosis stimulated by camptothecin. Consistent with these studies pretreatment of fibroblasts with *P. gingivalis* reduced TNF-α stimulated apoptosis that can be explained in part, by the downregulation of caspase-3. Thus, *P. gingivalis* has a unique ability to suppress caspase-3-dependent apoptosis while at the same time exerting a pro-

apoptotic effect through an alternative pathway. To investigate a caspase-independent pathway we examined AIF. Incubation of fibroblasts with *P. gingivalis* at an infective moi stimulated AIF translocation to the nucleus when measured by both confocal microscopy and immunoblot analysis. When AIF was downregulated by AIF siRNA, *P. gingivalis*-induced cell death was reduced by almost 50%. A second approach using a PKA inhibitor (H-89) that inhibits AIF activation (Park *et al*., 2005) reduced *P. gingivalis*-induced cell death by approximately 50%. This finding is consistent with a non-caspase-dependent mechanism reported for *Pneumococcus*-induced neuronal cell apoptosis (Braun *et al*., 2001). Thus, *P. gingivalis* may use a similar pathway to induce fibroblast cell death. However, it should be noted that not all apoptosis was inhibited by AIF siRNA or PKA inhibitor indicating that there likely to be another non-caspase-dependent pathway involved.

Experimental procedures

Cell culture conditions

Human gingival fibroblasts (HGF-1) were purchased from the American Type Culture Collection, ATCC (Manassas, VA). The cells were grown in fibroblast growth medium (FGM2) supplemented with 10% fetal bovine serum (Cambrex-Research Products, Pittsburgh, PA). For most assays cells from passages 5–10 were seeded in 24-well plates at 90% confluency (approximately 70 000 cells/well) overnight prior to starting experiments.

Porphyromonas gingivalis *growth conditions*

Porphyromonas gingivalis W83 was purchased from the American Type Culture Collection (ATCC). The bacteria were grown in a liquid broth of brain heart infusion, BHI, supplemented with hemin and menadione (Becton Dickinson, Sparks, MD) in an anaerobic chamber with GasPack containing 10% CO_2, 10% H2 and 80% N_2 (Becton Dickinson) for 1 day at 37°C. For each assay bacterial numbers were established by the number of colony-forming units of *P. gingivalis* serially diluted on blood agar plates (BD, Franklin Lake, NJ). In some experiments proteins released by *P. gingivalis* were tested for their effects on cell rounding and apoptosis. Culture supernatant from *P. gingivalis* W83 was filtered through 0.22 μM membrane, precipitated in ammonium sulphate (75% saturation), and chilled in −20°C for 30 min. Precipitated proteins were collected by centrifugation at 10 000 *g* for 20 min, and resuspended in phosphate-buffered saline (PBS). Protease activity was determined using N-benzoyl-DL-arginine-p-nitroanilide (BApNA) and N-acetyl-lysine-p-nitroanilide (AcKpNA) (Sigma-Aldrich, St. Louis, MO) as described by (Rangarajan *et al*., 1997).

Apoptosis and necrosis assays

Apoptosis was measured in human gingival fibroblasts using 24 well plates incubated in serum free media. In most experiments cells were incubated with *P. gingivalis* (moi 300:1). In some experiments cells were incubated with TNF-α (20 ng ml^{-1}) (R and D systems) plus cycloheximide (5 μg ml^{-1}), proteolytic extract (25 units ml^{-1}), or arginine and lysine specific endopeptidases (25 units ml^{-1}) (Geno Tech, St. Louis, MO). To evaluate the role of *P. gingivalis* proteases, inhibitors of Arg-gingipain or Lys-gingipain consisting of leupeptin (100 μM) or cathepsin B inhibitor II (100 μM) were added to the cells along with bacteria. It has been reported that leupeptin inhibits *P. gingivalis* RgpA/B but not Kgp (Houle *et al*., 2003; Andrian *et al*., 2004). Cathepsin B inhibitor II has been used to inhibit Kgp protease of *P. gingivalis* but does not inhibit RgpA/B (Houle *et al*., 2003). In order to block caspase activity human gingival fibroblasts were pretreated with the pancaspase inhibitor Z-VAD-fmk (100 nM) (EMD Biosciences) for 1.5 h before treatments with *P. gingivalis* or TNF-α. Cells were also preincubated with H-89 (50 μM) (EMD Biosciences) a selective inhibitor of protein kinase A (Chijiwa *et al*., 1990; Muniz *et al*., 1996; Muniz *et al*., 1997) before *P. gingivalis* treatment. Apoptosis of fibroblasts was assessed by ELISA measuring cytoplasmic histone-associated-

DNA fragments (Roche Applied Science, Indianapolis, IN) as described (Alikhani *et al*., 2005). Necrosis was measured by lactose dehydrogenase (LDH) released into cell culture media following centrifugation at 400 *g* for 10 min using a CytoTox 96 cytotoxicity assay kit (Promega, Madison, WI). In some experiments images were captured using an inverted light microscope equipped with digital camera. All experiment described were carried out a minimum of three times with similar results.

Caspase-3 measurements

Human gingival fibroblasts grown in 6-well plates were treated with *P. gingivalis* or TNF-α as above and total RNA was extracted using an RNAeasy kit (Qiagen, Valencia, CA). The RNA was converted to cDNA with MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) and caspase-3 mRNA levels were measured by real-time PCR using Taqman primers and probes (Applied Biosystems). mRNA levels are normalized to GAPDH mRNA and calculated relative to the unstimulated cells as by the manufacturer's guideline. To assay for caspase-3 enzyme activity a CaspGlo-3/7 Assay kit was used (Promega). Following the manufacturer's instruction, cells grown in 24-well plates and incubated with *P. gingivalis* for the indicated amount of time. In some experiments cells were preincubated with *P. gingivalis* for 45 min, rinsed thoroughly with PBS containing antibiotic and then stimulated with TNF-α (20 ng ml^{-1}). Cells were lysed and caspase-3 activity was measured in each well by luminescence assay. A BCA assay (Bio-Rad) was performed to ensure that an equal amount of cell protein was present in each well.

TUNEL assay

Human gingival fibroblasts with or without bacterial treatments were washed and fixed in 4% paraformaldehyde in PBS for 30 min at 4°C and washed three times with PBS. Cells were stained by an *in situ* terminal dUTP nick-end labelling (TUNEL) assay by means of a TACS 2 TdT-Blue Label kit purchased from Trevigen (Gaithersburg, MD). Cells showing apoptotic stain were counted using inverted microscope.

Immunofluorescence study

Human gingival fibroblasts with or without *P. gingivalis* treatment were fixed using 4% paraformaldehyde in PBS for 30 min at 4°C and washed three times with PBS. For the detection of AIF translocation, cells were permeablized with 1% Triton X-100 in PBS for 5 min at room temperature and incubated for 1 h with rabbit anti-AIF polyclonal antibody (BD Biosciences). Cells were washed three times as before and incubated with FITC conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) for additional 1 h and then counterstained with propidium iodide. Fluorescence staining was detected using a confocal laser microscope (Axiovert, Carl Zeiss, Thornwood, NY) and positive cells showing nuclear translocation of AIF were counted. This experiment was performed three times with similar results.

siRNA study

To evaluate the role of AIF we used small interfering RNA (siRNA) that blocks the expression of this gene. Silencing *aif* gene expression in human gingival fibroblasts was achieved by using siRNA purchased from (Qiagen). This experiment was performed for the most part following the manufacturer's guideline. Briefly, cells grown in 6- or 24-well plates were transfected with AIF or scrambled siRNA using HiPerFect transfection reagent (Qiagen) for 24 h when they were approximately 70% confluent. Cells were then rinsed and incubated in standard cell culture medium for an additional 24 h. Cells were then incubated with *P. gingivalis* after which they were assayed for AIF mRNA level or for presence of AIF in the nucleus using immunoblotting.

Immunoblots

Human gingival fibroblasts incubated with or without *P. gingivalis* were lysed for extraction of nuclear proteins using a nuclear extraction kit (Pierce, Rockford, IL). Briefly, 10 μg of nuclear extracts were separated on SDS-PAGE (12% polyacrylamide) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen). The membrane was blocked with skim milk and washed before incubating with specific antibodies. The primary antibodies used in immunoblotting were rabbit antiserum against AIF (Pharmingen) and goat antiserum against actin, a cytoplasmic protein marker or lamin-B, a nuclear protein marker (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies were donkey anti-rabbit or anti-goat conjugated with horseradish per-oxidase (HRP) (Santa Cruz Biotechnology). Immunoblots were developed using enhanced chemiluminescence reagents (Santa Cruz). When necessary, the blots were stripped with a Western blot stripping reagent (Pierce) for a second use.

Statistics

Statistical significance was determined by one way analysis of variant (ANOVA). Unless otherwise stated the values from three separate experiments were combined to obtain the mean and standard error of the mean. Significant differences were established at $P < 0.05$.

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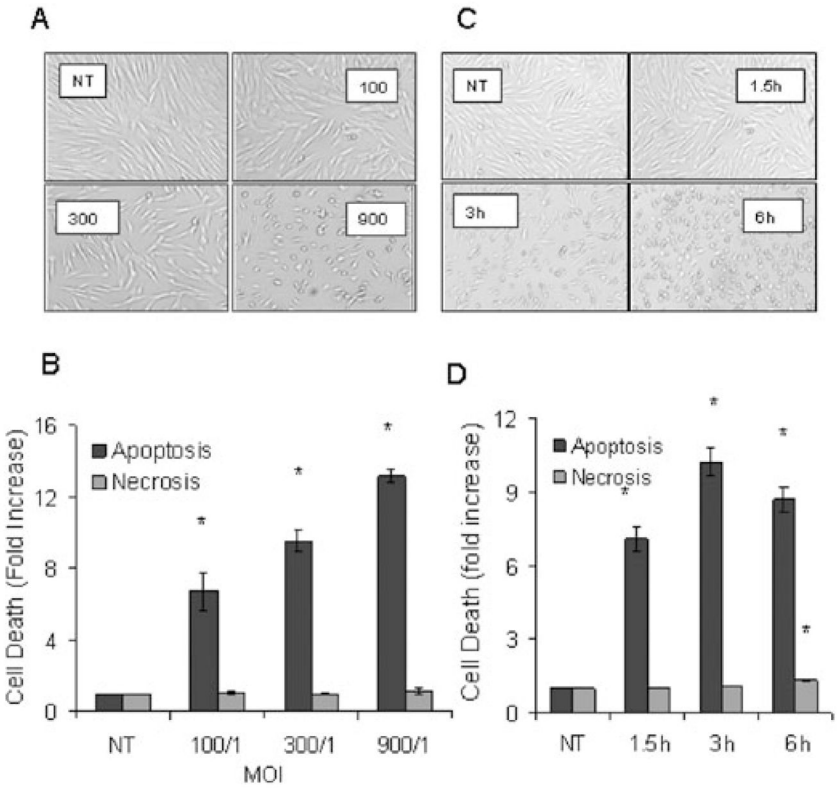

Fig. 1.

P. gingivalis induces a dose- and time-dependent increase in fibroblast apoptosis but not necrosis that is distinct from cell rounding.

A. Human gingival fibroblasts were incubated with *P. gingivalis* (moi 100:1, 300:1 and 900:1) for 3 h. Original magnifications 40×.

B. Cells from A were tested for apoptosis by the presence of cytoplasmic histone-associated DNA fragments by ELISA (dark bars) or for necrosis by the release of LDH (light bars).

C. Human gingival fibroblasts were incubated with *P. gingivalis* (moi 300:1) for 1.5, 3 or 6 h. Original magnifications 40×.

D. Cells from C were tested for apoptosis (dark bars) and necrosis (light bars) as described above. Each value represents the mean of three separate experiments ± SEM. An asterisk indicates significant increase ($P < 0.05$).

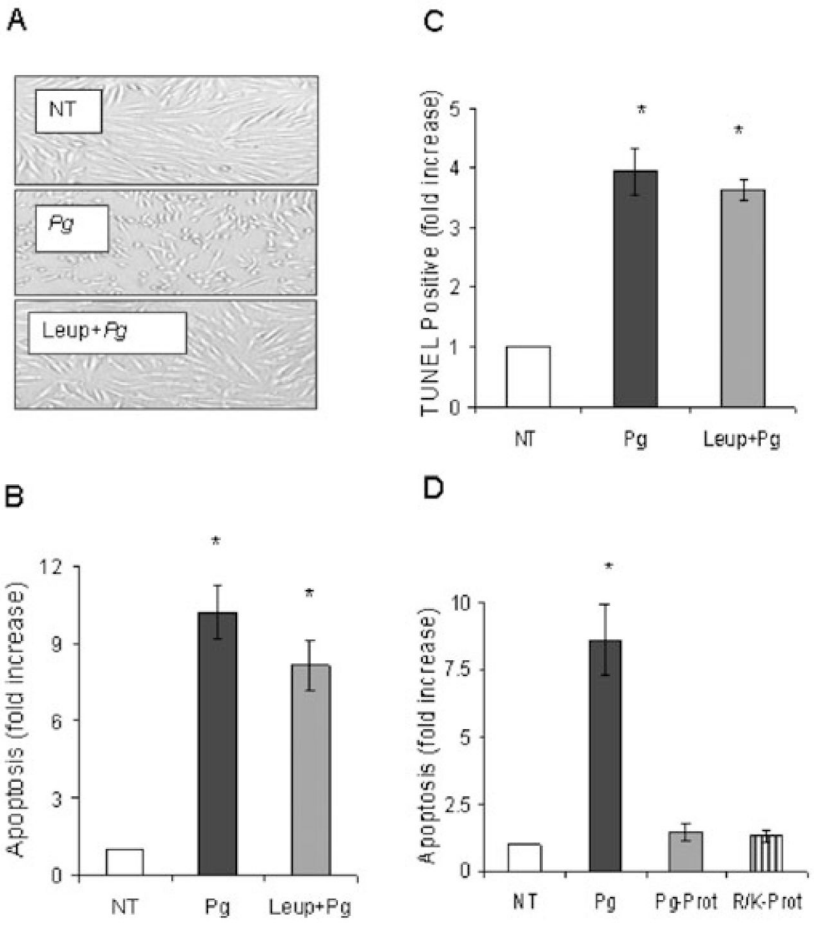

Fig. 2.

P. gingivalis induces fibroblast apoptosis independent of its proteolytic enzymes. Human gingival fibroblasts were exposed to *P. gingivalis* (moi 300:1) with or without leupeptin for 3 h.

A. Original magnifications 40×.

B. Apoptosis was measured by ELISA.

C. The TUNEL assay was performed and TUNEL positive cells were counted.

D. Human gingival fibroblasts were also exposed to *P. gingivalis*-supernatant (*Pg*-prot) or arg/lys-specific endopeptidases (R/K-prot). Apoptosis was measure by ELISA. Each value represents the mean of three separate experiments ± SEM. An asterisk indicates significant increase ($P < 0.05$).

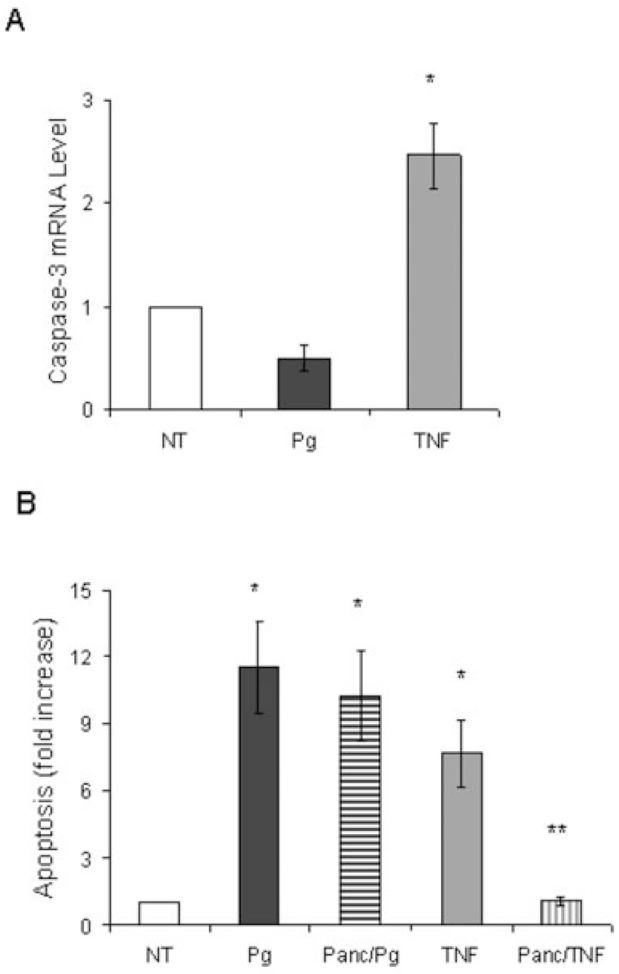

Fig. 3.

P. gingivalis-induced fibroblast apoptosis is caspase-independent.

A. Human gingival fibroblasts were incubated with *P. gingivalis* or TNF and caspase-3 mRNA levels were determined by real-time PCR.

B. Cells were preincubated with or without a pan-caspase inhibitor (Panc) for 1.5 h, rinsed thoroughly and then incubated with *P. gingivalis* or TNF for 3 h. Apoptosis was measured by ELISA as before. Each value represents the mean of three separate experiments ± SEM. The single asterisk indicates significant increase and the double asterisks indicate significant decrease ($P < 0.05$).

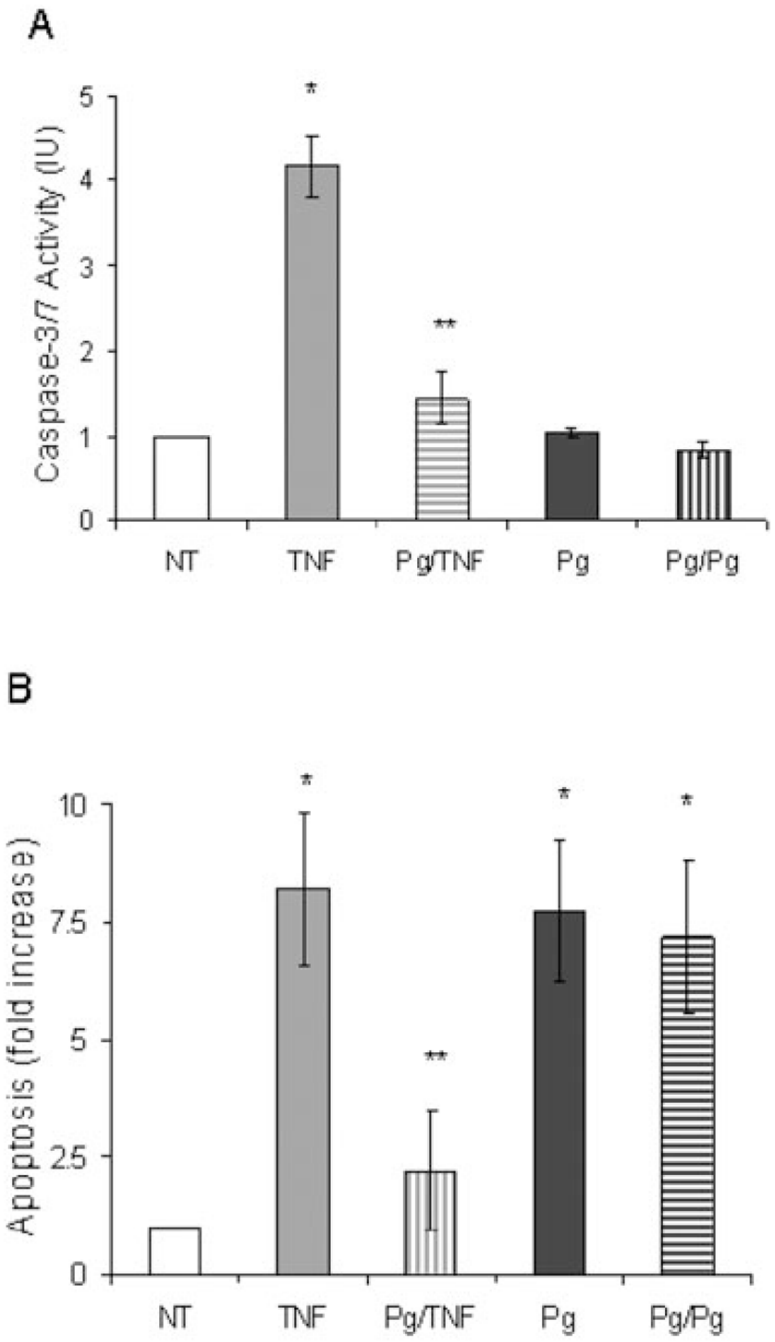

Fig. 4.

Pretreatment of fibroblasts with *P. gingivalis* downregulates caspase-3 activity and TNF-induced apoptosis. Cells were preincubated with *P. gingivalis* for 45 min and rinsed with PBS containing antibiotic. Following rinses cells were incubated in serum free media containing *P. gingivalis* (moi 300:1) or TNF (20 ng ml^{-1}) for additional 3 h.

A. Caspase-3 activity was measured.

B. Apoptosis was measured by ELISA. Each value represents the mean of three separate experiments ± SEM. The single asterisk indicates significant increase and the double asterisks indicate significant decrease ($P < 0.05$).

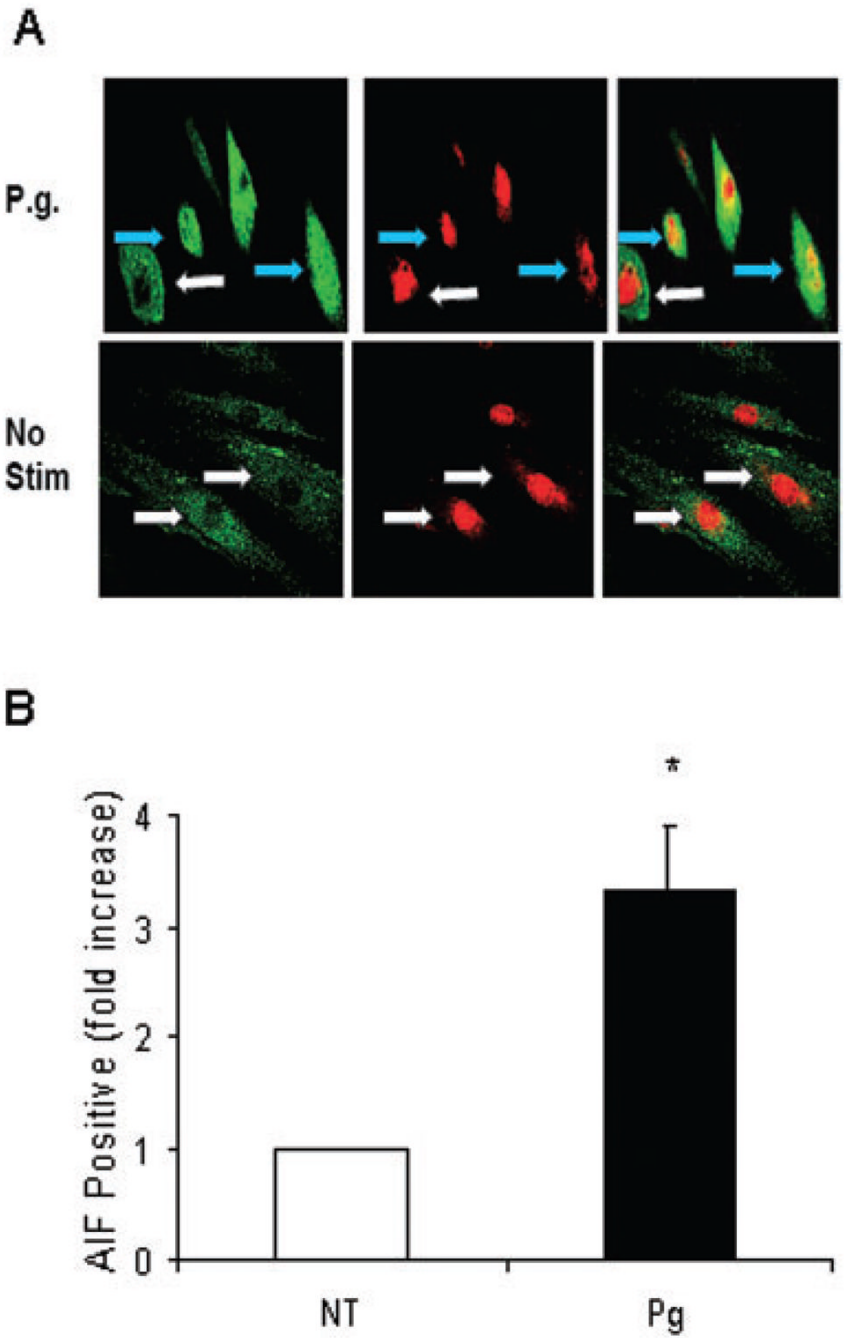

Fig. 5.

P. gingivalis Induces Nuclear Translocation of AIF.

A. Human gingival fibroblasts treated with or without *P. gingivalis* were immunostained with antibody to AIF using an FITC (green) detection system and counterstained with propidium iodide (red) to identify the nuclear compartment. Yellow stain in the upper right panel identifies cells with AIF in the nuclear compartment. Blue arrows indicate nuclear AIF and the absence of AIF in the nucleus is designated by white arrows.

B. Cells positive for AIF nuclear translocation were quantified.

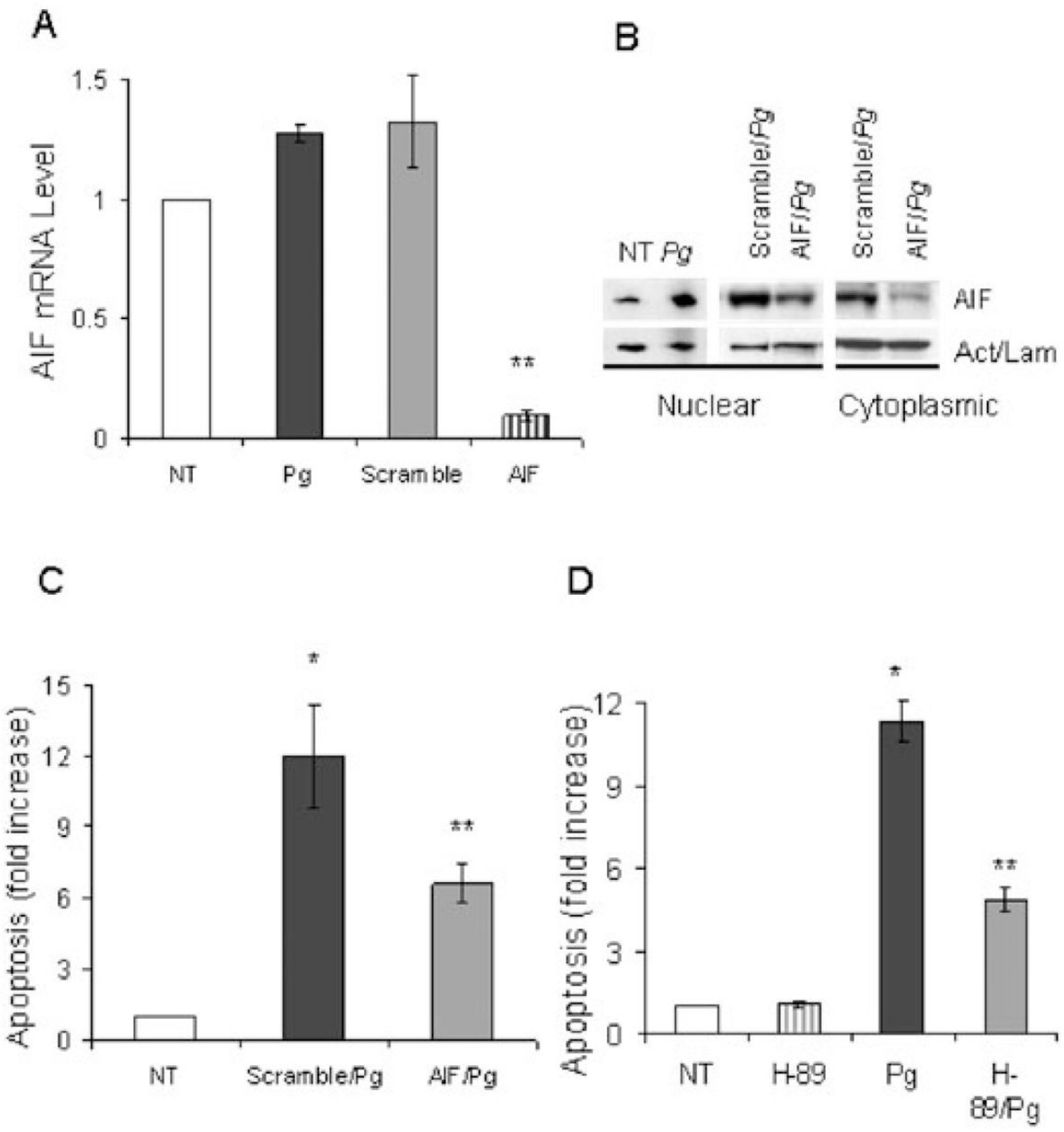

Fig. 6.

Silencing AIF gene reduces *P. gingivalis*-induced fibroblast apoptosis. Human gingival fibroblasts plated at 70% confluency were preincubated with AIF-siRNA (AIF) or scrambled-siRNA (Scramble) for 24 h. Cells were left for additional 24 h with regular media and incubated with *P. gingivalis* for 3 h.

A. AIF mRNA levels were measured by real-time PCR.

B. Nuclear and cytoplasmic proteins extracted from cells described in A. Blots were incubated with antibody to AIF stripped and then incubated with antibody against actin for cytoplasmic or lamin B for nuclear proteins.

C. Apoptosis was measured by ELISA.

D. Human gingival fibroblasts were preincubated with H-89 for 1 h, washed, and incubated with bacteria for 3 h. Apoptosis was measured by ELISA. Each value represents the mean of three separate experiments ± SEM. The single asterisk indicates significant increase and the double asterisks indicate significant decrease ($P < 0.05$).