Caspase activation is involved in chronic periodontitis

Heike Bantel a, Thomas Beikler b, Thomas F. Flemmig b, Klaus Schulze-Osthoff a,* a Institute of Molecular Medicine, University of Düsseldorf, Universitätstrasse 1, Building 23.12, D-40225 Düsseldorf, Germany
b Clinic of Periodontology, University of Münster, D-48147 Münster, Germany

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Abstract  Periodontitis, a common infectious disease, is initiated by various gram-negative bacteria and characterized by the destruction of the periodontal tissue. Here, we investigated the role of caspases, intracellular proteases that are the key mediators of apoptosis. We show that activation of caspase-3 and caspase-7 is considerably enhanced in gingival tissue from patients with periodontitis. We also demonstrate in in vitro experiments that various periodontopathic bacteria exert a direct growth-suppressing effect and, moreover, can trigger a host-mediated cytotoxic activity involving the CD95 death receptor. Our data suggest that caspase activation is a prominent feature in periodontitis-associated tissue injury.

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1. Introduction

Periodontitis is a common infectious disease characterized by inflammation and destruction of periodontal tissue which can result in tooth loss [1]. The severity of periodontitis correlates with the presence of certain bacteria that mediate inflammatory responses and cause periodontal tissue destruction. Various periodontal pathogens including Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Eikenella corrodens have been identified. The mechanisms responsible for gingival tissue damage are poorly understood, and both immune-mediated reactions and direct cytopathic effects of bacteria may be involved. Based on a direct effect of bacteria in cell cultures, it has been suggested that apoptosis might play an important role in periodontitis [2–6]. However, it remains unknown which molecular mechanisms participate in this process.

Recent studies demonstrate that apoptosis is essentially mediated by a family of cysteine proteases, called caspases, which can be divided into initiator and effector caspases [7]. Initiator caspases, such as caspase-8 or -9, exert regulatory roles by activating downstream effector caspases, such as caspase-3, -6, or -7, which cleave various cellular substrates [8]. Activation of caspases is achieved via two signaling pathways [9]. The extrinsic death pathway involves the ligation of death receptors that leads to the recruitment of pro-caspase-8 into a death-inducing signaling complex. The intrinsic death pathway is initiated by the mitochondrial release of cytochrome c, a process that is inhibited by anti-apoptotic Bcl-2 proteins. When released, cytochrome c binds together with the apoptosis protease-activating factor-1 to procaspase-9 to form the apoptosome.

One of the best-defined apoptotic pathways is mediated by the death receptor CD95, which is expressed on many cell types including gingival and skin keratinocytes [10–12]. The CD95 pathway is triggered upon binding of its ligand CD95 ligand (CD95L) that is expressed mostly in T-cells but also in other cell types. T-cells that recognize bacterial antigen become activated and indubitably express CD95L that can then transduce a death signal into gingival keratinocytes and other CD95-bearing cells [10].

Despite the elucidation of apoptotic signaling cascades, it is almost completely unknown whether and to which extent caspases are activated in human pathologies. In the present study, we investigated the activation of effector caspases in gingival biopsies from patients with chronic periodontitis. We demonstrate that periodontitis-associated tissue damage is characterized by a strongly increased activation of caspases. We also provide evidence that periodontopathic bacteria can trigger a host-mediated cytotoxic activity involving at least partially the CD95 death receptor system.

2. Materials and methods

2.1. Gingival biopsies

Eighteen patients (8 males, 10 females; 22–66 years) with previously untreated moderate to severe periodontitis, and 11 healthy control individuals (7 males, 5 females, 28–37 years) were recruited. Subjects in the control group had no prior history of periodontal disease and had no signs of attachment loss or clinical inflammation. Gingival biopsies were obtained under local anaesthesia and consisted of oral gingival epithelium, pocket epithelium and the underlying connective tissue. Biopsies from controls were taken from the premolar area on the palate using a paramarginal incision close to the gingival margin.

2.2. Immunoblotting and fluorimetric determination of caspase activation

Gingival biopsies were homogenized in 1% Triton X-100, 50 mM Tris–HCl, pH 7.6 and 150 mM NaCl containing 3 μM aprotinin, 3 μg/ml leupeptin, 3 μg/ml pepstatin A and 2 mM phenylmethylsulfonyl fluoride. After centrifugation, homogenates were separated by 15% SDS–PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were immunoblotted with monoclonal antibodies against caspase-3 (Transduction Laboratory, Heidelberg, Germany) or caspase-7 (Pharmingen, Hamburg, Germany) as described [13]. Caspase activity was also determined by incubation of tissue

Abbreviations: CD95L, CD95 ligand; MNC, mononuclear cell; PARP, poly(ADP-ribose)polymerase.
homogenates with the fluorogenic substrate Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aminomethyl-coumarin; Bachem, Heidelberg, Germany). To this end, homogenates were incubated with 50 μM DEVD-AMC in 200 μl buffer containing 50 mM HEPES pH 7.4, 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 mM dithiothreitol, and measured in a fluorimeter using an excitation and emission wave-lengths of 360 and 475 nm.

2.3. Immunohistochemical staining of tissue sections
Frozen sections (5 μm) of gingival tissue were examined. After blocking endogenous peroxidase and non-specific binding the activation-specific anti-caspase-3 (0.3 μg/ml, RDS, Wiesbaden, Germany), anti-caspase-7 (1 μg/ml, NEB, Beverly, MA) and anti-poly(ADP-ribose)-polymerase (PARP) antibodies (0.2 μg/ml) were added to the slides. The characterization of the anti-caspase and the cleavage site-directed anti-PARP antibodies has been described previously[13,14]. Isotype-matched control antibodies served as a control. The reactions were developed using biotinylated horse anti-rabbit IgG and peroxidase-conjugated avidin–biotin complex (Vector Laboratories Burlingham, CA). Finally, the sections were stained with amino-9-ethyl-carbazole and counterstained with hematoxylin. The number of immunoreactive cells was assessed by an imaging software. Positive cells in four microscopic fields of a 400-fold magnification were counted. Statistical analysis was performed using the U-test according to Mann and Whitney. A P-value <0.001 was considered to be significant.

2.4. Bacterial coincubation and cell death assays
*Actinobacillus actinomycetemcomitans* ATCC 33384, *E. corrodens* BCMG 232 and *P. gingivalis* MCCM 527 were incubated under anaerobic conditions at 37°C in M199 medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS and 10% horse serum without antibiotics. Bacterial coincubation assays were performed with primary human gingival fibroblasts, the immortalized keratinocyte cell line HaCaT and peripheral blood mononuclear cells (MNCs). Gingival fibroblasts and HaCaT cells were cultured in complete M199 medium. MNCs were prepared by Ficoll gradient centrifugation and maintained in RPMI 1640 medium supplemented with 10% FCS.

In order to assess direct cytotoxic effects of the bacteria, gingival fibroblasts and HaCaT cells were seeded with 3 ml growth medium into 6-well plates at a density of 5 × 10^5 cells/well. Prior to coincubation, cells were washed and then incubated with *A. actinomycetemcomitans* (1.4 × 10^7–4.2 × 10^7 CFU/well), *E. corrodens* (1.5 × 10^7–7.4 × 10^7 CFU/well) and *P. gingivalis* (1.2 × 10^7–1.8 × 10^8 CFU/well). Then, the bacteria were removed and the cells were further incubated in medium containing amphotericin B (0.5 mg/l) and gentamycin (96 mg/l). Following incubation for various times (6–72 h), cells were fixed with acetone–methanol (1:1). Cell death was quantified by the crystal violet method. Following incubation for various times (6–72 h), cells were fixed with acetone–methanol (1:1). Cell death was quantified by the crystal violet method. Following incubation for various times (6–72 h), cells were fixed with acetone–methanol (1:1). Cell death was quantified by the crystal violet method.

3. Results

3.1. Immunoblot and enzymatic analysis of caspase activation in periodontitis-associated gingival tissues
In initial experiments, we investigated the activation of caspase-3 and -7, two essential effector caspases, by monitoring the processing of their proforms in immunoblot analyses. Homogenates of healthy gingival tissue as well as of biopsies from patients with chronic periodontitis were prepared and analyzed using antibodies specific to the individual proteases. As shown in Fig. 1A, in gingival biopsies of healthy controls only the 32 kDa form of procaspase-3 was detected, whereas the active subunits were almost not visible. In contrast, in chronic periodontitis tissue the conversion of procaspase-3 into its 21 kDa intermediate fragment and the 17 kDa active subunit was clearly evident. Similarly, an antibody against caspase-7 detected only the inactive p32 precursor form of caspase-7 in healthy individuals, whereas periodontitis samples revealed also a 20 kDa protein band, corresponding to the large active subunit of caspase-7 (Fig. 1B). To further verify the elevated caspase activation in periodontitis patients, we performed caspase activity assays using the fluorogenic caspase-3 substrate DEVD-AMC. As demonstrated in Fig. 2, tissue homogenates from patients with chronic periodontitis contained DEVDase activity that was significantly higher than the activity in normal healthy gingival tissue. Thus, these data indicate that caspase activation is increased in chronic periodontitis.

3.2. In situ detection of caspase activity and PARP cleavage in biopsies with chronic periodontitis
Next we analyzed gingival tissue sections by immunohistochemistry using activation-specific antibodies against active caspase-3 and caspase-7. By a similar approach, we also determined the cleavage of poly(ADP-ribose)-polymerase (PARP), a DNA repair enzyme that is cleaved by caspase-3. The antibodies used were specific for the proteolytically generated neoepitopes and active forms of the molecules, but did not recognize the inactive proforms of either caspase or non-cleaved PARP, respectively[13,14]. Fig. 3A–C shows that almost no immunoreactivity was evident in healthy gingival tissue. In contrast, chronic periodontitis tissue clearly showed many cells that stained positively for active caspase-3, caspase-7 as well as

![Image](521x214 to 539x240)

Fig. 1. Immunoblot analysis of caspase activation in periodontitis. Gingival homogenates from a healthy control (C) and a patient with periodontitis (P) were immunoblotted with anti-caspase-3 (A) and anti-caspase-7 (B) antibodies. The inactive proforms of the caspases are marked with an open arrowhead, and the processed fragments with a closed arrowhead. In homogenates of chronic periodontitis procaspase-3 was cleaved into the 21 and 17 kDa fragments, whereas in gingival tissue from a healthy control almost no caspase-3 processing was observed. Similarly, healthy controls contained exclusively the proform of caspase-7, whereas periodontitis patients additionally expressed the active p20 subunit of caspase-7.
performed coincubation experiments of HaCaT keratinocytes with periodontopathic bacteria or indirect host- and immune-mediated mechanisms, we have shown that periodontitis might be caused by a direct cytotoxic effect of bacteria. 

3.3. Periodontopathic bacteria induce host-mediated cytotoxic effects

To investigate whether the increased caspase activation in periodontitis tissue is caused by the bacteria, at least in vitro, we performed coincubation experiments of HaCaT keratinocytes and primary gingival fibroblasts with different periodontopathic bacteria. Incubation of keratinocytes with Actinomyces actinomycetemcomitans, E. corrodens and P. gingivalis caused an approximately 20–30% reduction of cell viability (Fig. 5A). A slightly weaker effect was observed in gingival fibroblasts (Fig. 5B). A closer inspection of the cell cultures, however, revealed that this effect was entirely due to a growth suppression caused by the bacteria. Moreover, incubation of the cells did not induce caspase activation or apoptotic alterations, such as membrane blebbing, cell shrinkage or chromatin condensation (data not shown). In contrast, staurosporine, a classical proapoptotic agent, strongly induced apoptosis in both gingival fibroblasts and HaCaT cells (Fig. 5A and B). Both cell types also underwent apoptosis when incubated with agonistic anti-CD95 antibody in the presence of cycloheximide. Induction of apoptosis was also found when fibroblasts and keratinocytes were coincubated with K562 cells that overexpressed membrane-bound CD95L, which induces efficient death receptor crosslinking (Fig. 5A and B).

Since certain bacteria can induce apoptosis by upregulation of CD95L, we next investigated whether cytotoxicity could be indirectly triggered by such a host-mediated mechanism. To this end, blood mononuclear cells (MNCs) were infected with the bacteria, and further coincubated with HaCaT keratinocytes 24 h postinfection. As shown in Fig. 5C, infection with Actinobacillus, Eikenella, and to a weaker extent with Porphyromonas induced a cytotoxic activity in MNCs against HaCaT target cells. Interestingly, the cytotoxic activity of the MNCs was partially, but consistently abolished by neutralizing anti-CD95L antibodies. These results therefore suggest that periodontal bacteria, at least in vitro, exert growth-suppressing effects and can trigger apoptotic events by host-mediated mechanisms involving the CD95 death receptor.

4. Discussion

Data from in vitro cell cultures and a few reports of patient biopsies had suggested that apoptosis might play a role in periodontitis-associated gingival tissue damage [2–6,17]. However, the relative contribution of apoptosis and the functional role of caspases in periodontal tissue damage remained largely unknown. In the present study, we demonstrate by immunoblot and substrate assays that caspase-3 and -7, two major effector caspases, are activated to a higher extent in tissue homogenates from patients with chronic periodontitis than in healthy tissue. Furthermore, increased caspase activation was directly detectable in inflamed gingival biopsies. A considerable number of cells in the gingival epithelium and connective tissue revealed active caspases, whereas in healthy tissue almost no caspase activation was observed. These results therefore suggest that caspase activation may be functionally involved in periodontitis-associated tissue damage.

Previously, the occurrence of apoptosis in chronic periodontitis has mainly been studied by morphologic criteria as well as by the widely used TUNEL technique that is based on the detection of DNA fragmentation. An increased number of TUNEL-positive inflammatory cells has been reported in periodontitis [17]. By using three independent markers, i.e., caspase-3 and -7 activation as well as PARP cleavage, our results clearly show caspase activation is found in keratinocytes, basal and connective tissue of the gingiva.
cells and connective tissue cells, cell types that are damaged in periodontitis. This finding is compatible with a study demonstrating that cells with p53 expression and DNA damage are mainly localized in the epithelium and connective tissue of periodontitis patients [5].

The rate of cells showing caspase activation was considerably high with all three apoptotic markers, even though the cells often revealed no classical apoptotic alterations. This high number of cells with active caspases certainly exceeds the number of cells that can be usually detected by techniques measuring DNA fragmentation, which is a late event in apoptosis [13]. There are several possibilities to explain this finding. It is commonly assumed that activation of caspases results in apoptosis, although there is increasing evidence that within a cell the extent of caspase activation may be restricted and not necessarily lead to cell death [18–21]. More importantly is presumably the fact that caspase activation is a very early event in apoptosis. Cells in later stages might be rapidly phagocytosed by macrophages and therefore escape morphological detection.

Several bacteria are able to trigger apoptosis of host cells either by direct or indirect mechanisms [22]. Direct effects can be mediated by different virulence factors, whereas indirect processes are mostly immune-mediated. It has been shown that *A. actinomycetemcomitans* induces apoptosis in cultured epithelial cells and macrophages, a process that may be mediated by leukotoxin or other bacterial factors [3,6]. *P. gingivalis* has been reported to induce apoptosis in T-cell lines [5]. In addition, proteases of *P. gingivalis* such as gingipains can induce the loss of attachment of fibroblasts, which could mediate cell death by anoikis [23,24]. In support of the present data, it was...
Periodontopathic bacteria induce a cytotoxic activity in blood mononuclear cells that partially involves CD95. Mononuclear cells were overexpressing membrane-bound CD95L. After 36 h cell survival was measured by the crystal violet assay in triplicate cultures and calculated as the percentage relative to the untreated controls. (C) Periodontopathic bacteria induce a cytotoxic activity in blood mononuclear cells that partially involves CD95. Mononuclear cells were infected with the bacteria as described above. 24 h later the bacteria were removed, and the mononuclear cells (1.6 x 10^4/well) were added to the HaCaT target cells. Some HaCaT cultures were additionally incubated with neutralizing anti-CD95L (20 µg/ml) or control IgG, and cell death was assessed after further 36 h.

In our experiments gingival fibroblasts and HaCaT keratinocytes were almost completely resistant to soluble agonistic anti-CD95 antibodies. CD95L when expressed in the cell surface, in contrast, was more potent in inducing apoptosis due to efficient crosslinking of the CD95 receptor. Thus, both cell types are principally sensitive to CD95-triggered apoptosis, although their sensitivity is less pronounced than in other cell types, e.g., T-lymphocytes. Consequently, we investigated whether fibroblasts and keratinocytes could be indirectly killed by the bacteria through the induced upregulation of CD95L or other proapoptotic stimuli in peripheral blood MNCs. After bacterial infection of MNCs and subsequent coincubation with HaCaT cells, we observed that A. actinomycetemcomitans and E. corrodens induced a cytotoxic activity in MNCs that was interestingly attenuated by neutralizing anti-CD95L antibodies. Whether the remaining cytotoxic activity induced in bacteria-treated MNCs is due to other death ligands or unrelated cytotoxic mediators remains to be clarified. Thus, our data indicate that host-mediated mechanisms are involved in periodontitis-associated tissue damage. Moreover, the finding that tissue injury is associated with elevated caspase activation could open up new diagnostic possibilities and therapeutic strategies to prevent tissue destruction in periodontal disease.

Fig. 5. Effect of periodontopathic bacteria on cell viability. Gingival fibroblasts (A) and HaCaT keratinocytes (B) were seeded into 6-well plates and infected with Actinobacillus actinomycetemcomitans (Aa, 4.2 x 10^7 CFU/well), Eikenella corrodens (Ec, 7.4 x 10^7 CFU/well) and Porphyromonas gingivalis (Pg, 1.8 x 10^8 CFU/well). As a positive control cells were treated with staurosporine (Stau) or anti-CD95 in the presence or absence of cycloheximide (CHX). CD95-mediated apoptosis was also assessed by coincubation of target cells with K562 cells overexpressing membrane-bound CD95L. After 36 h cell survival was measured by the crystal violet assay in triplicate cultures and calculated as the percentage relative to the untreated controls. (C) Periodontopathic bacteria induce a cytotoxic activity in blood mononuclear cells that partially involves CD95. Mononuclear cells were infected with the bacteria as described above. 24 h later the bacteria were removed, and the mononuclear cells (1.6 x 10^4/well) were added to the HaCaT target cells. Some HaCaT cultures were additionally incubated with neutralizing anti-CD95L (20 µg/ml) or control IgG, and cell death was assessed after further 36 h.

also reported that *P. gingivalis* did not directly induce, but even inhibited apoptosis [25,26]. It must be noted that most studies demonstrating a proapoptotic effect of periodontitis-associated bacteria employed cell lines that, unlike keratinocytes, are highly sensitive to apoptosis.

In keratinocytes and fibroblasts we did not observe a direct apoptotic effect of the bacteria, suggesting that rather immune or host-mediated reactions are involved in caspase activation. Important mediators in immune-mediated tissue damage are death receptors, in particular CD95. In many inflammatory conditions CD95L expression is induced in antigen-stimulated T-cells. CD95L may additionally exert proinflammatory activities by inducing IL-1β secretion and neutrophil infiltration [27]. CD95 is expressed in gingival epithelial cells from chronic periodontal lesions [11]. Thus, induction of CD95L expression in lymphocytes or other cells in response to bacterial infection may trigger caspase activation in gingival epithelial cells.

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