Role of caspases in cleavage of lamin A/C and PARP during apoptosis in macrophages infected with a periodontopathic bacterium

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The periodontopathic bacterium Actinobacillus actinomycetemcomitans has been implicated in the pathogenesis of periodontal diseases. It has been reported previously that infection with the organism induced apoptosis in the mouse macrophage cell line J774.1. In the present study, the role of caspases during apoptosis in A. actinomycetemcomitans-infected J774.1 cells was examined. A large number of apoptotic cells was detected by flow cytometric analysis in infected J774.1 cells; however, inhibitors of caspase-9, -6 and -3/7 completely blocked the induction of apoptosis. Expression of the cleaved forms of caspase-6 and -7 was detected during apoptosis in infected J774.1 cells. Immunoblot analysis revealed that the caspase-9 inhibitor blocked expression of the cleaved forms of caspase-6 and -7, whilst the caspase-3 inhibitor blocked expression of the cleaved form of caspase-7, but not caspase-6. It is known that lamin A/C and poly(ADP-ribose) polymerase (PARP) are essential nuclear components for maintaining normal cell function and viability, and both were found to be cleaved in the infected J774.1 cells. Immunoblot analysis also revealed that the caspase-6 inhibitor blocked the cleavage of lamin A/C, whilst the caspase-3/7 inhibitor blocked the cleavage of PARP. Taken together, these results suggest that activation of caspases and the subsequent cleavage of lamin A/C and PARP are involved in the morphological changes of apoptotic macrophages infected with A. actinomycetemcomitans.

INTRODUCTION

Actinobacillus actinomycetemcomitans, a Gram-negative bacterium, has been recovered from periodontal tissues affected by periodontal disease (Christersson et al., 1987). It has also been reported that the organism is a causative agent of various infectious diseases, such as periodontitis, endocarditis, pericarditis, meningitis, osteomyelitis, emphysema, and subcutaneous abscesses (Kaplan et al., 1989; Nishihara & Koseki, 2004).

Direct in vitro evidence of invasion of human epithelial cells by A. actinomycetemcomitans has been reported (Sreenivasan et al., 1993), and it is known that invasion of the organism into scavenger cells by phagocytosis readily occurs. We previously developed an in vitro cell culture infection model using this bacterium to clarify the pathogenesis of periodontopathic bacteria for induction of apoptosis in macrophages (Kato et al., 1995).

Abbreviations: PARP, poly(ADP-ribose) polymerase; PI, propidium iodide.

Apoptosis has been shown to play an important role in the control of various biological systems, such as the immune response, haematopoiesis and embryonic development (Raff, 1992). The process involves active cell death and can be triggered by a variety of physical agents, as well as some infectious diseases (Raff, 1992; Zychlinsky et al., 1992). Caspases appear to be major effectors in the apoptotic process, with more than ten identified and partially characterized, several of which have been implicated in the induction of apoptosis (Cohen, 1997).

We reported previously that the release of cytochrome c from mitochondria, and activation of caspase-1, -3 and -9 are involved in the induction of apoptosis in macrophages infected with A. actinomycetemcomitans (Kasai et al., 2004; Nonaka et al., 2001). In the present study, we investigated the involvement of effector caspases in the induction of apoptosis in infected macrophages, using peptide inhibitors. We also clarified the role of nuclear components in the morphological changes in those apoptotic cells infected by A. actinomycetemcomitans.
METHODS

Cells and bacterial strain. The murine macrophage cell line J774.1 was obtained from the Japanese Cancer Research Resources Bank. J774.1 cells were cultured in RPMI 1640 (Gibco Laboratories) supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹) at 37 °C in an atmosphere of 5% CO₂ in air. A. actinomycetemcomitans Y4 was grown in brain heart infusion broth (Difco Laboratories) supplemented with 1% yeast extract at 37 °C for 3 days in an atmosphere of 5% CO₂ in air (Kato et al., 1995).

Reagents and antibodies. A caspase-3 inhibitor, benzoxycarbonyl-Asp-Val-Asp-OMe fluoromethyl ketone (Z-DEVD-FMK), and a caspase-9 inhibitor, benzoxycarbonyl-Asp-Val-Asp-OMe His-Asp-(OMe)-CH2F (Z-LEHD-CHO), were purchased from Calbiochem. A caspase-3/7 inhibitor, acetyl-Asp-Val-Asp-Asp-H (Ac-DEVD-CHO) was purchased from the Peptide Institute, Japan. A caspase-6 inhibitor, acetyl-Val-Glu-Ile-Asp-CHO (Ac-VEID-CHO), was purchased from Kamiya Biomedical Co. Monoclonal antibodies against lamin A/C, poly(ADP-ribose) polymerase (PARP) and β-actin were purchased from Santa Cruz Biotechnology. A monoclonal antibody against caspase-6 was purchased from Chemicon International. A monoclonal antibody against β-actin was purchased from Sigma.

Infection of J774.1 cells and culture conditions. J774.1 cells were cultured in tissue culture set-ups (100 cells ml⁻¹) and seeded in six-well plates (Corning Glass Works) at a concentration of 4 × 10⁵ cells ml⁻¹ 1 day prior to the beginning the experiment. A. actinomycetemcomitans Y4 organisms were harvested by centrifugation and suspended in RPMI 1640 without antibiotics to an OD₆₀₀ 0.4, which corresponded to approximately 2 × 10⁶ bacteria ml⁻¹. Bacterial suspension was added to the wells, and the plates were centrifuged at 1000 g for 10 min at 4 °C prior to incubation at 37 °C for 1 h. J774.1 cells infected with A. actinomycetemcomitans Y4 at a final bacterium : cell ratio of 5000 : 1 were washed three times with RPMI 1640 containing penicillin G (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and gentamicin (200 μg ml⁻¹) to remove extracellular bacteria. The infected J774.1 cells were cultured with RPMI 1640 containing 5% fetal bovine serum and antibiotics for 12, 18, 24, 30, 36 or 48 h (Kato et al., 1995), with or without caspase inhibitors (100 μM) (Nonaka et al., 2001).

Analysis of DNA content of apoptotic nuclei. To detect apoptotic nuclei, infected cells (4 × 10⁵) were suspended in hypotonic solution (0.1% sodium citrate, 0.2% NP-40, 0.25 mg RNase ml⁻¹, pH 8.0), stained with propidium iodide (PI) (50 μg ml⁻¹) and analysed using a FACScan (Becton Coulter). The cell-cycle phases were analysed using PC cyclic for Windows (Phoenix Flow System).

Immunoblot analysis. A. actinomycetemcomitans-infected J774.1 cells were lysed in SDS lysis buffer (50 mM Tris/HCl, pH 6.8, 2% SDS). The protein content of the samples was determined using a protein assay reagent (Bio-Rad). Each protein sample (20 μg per well) was then electrophoresed on 7.5, 12.5 or 15% SDS-polyacrylamide gels and electroblotted onto PVDF membranes. After incubation with 5% non-fat skimmed milk in PBS (pH 7.2) containing 0.1% Tween 20 for 1 h, the membranes were reacted with primary antibodies overnight at 4 °C. Immunodetection was performed using the ECL-Plus Western blotting detection system (Amersham Bioscience).

RESULTS AND DISCUSSION

PI-stained histograms can be used to clearly distinguish nuclei with normal diploid DNA from apoptotic nuclei with hypodiploid DNA (subG1 DNA) (Kasai et al., 2004). Apoptotic cells are recognized on the basis of their reduced DNA content and morphological changes, which include nuclear condensation and which can be detected by flow cytometry (subG1 DNA content), trypan blue or Hoechst staining (Oancea et al., 2006). The PI flow cytometric assay has been widely used for the evaluation of apoptosis in different experimental models. It is based on the principle that apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Use of a fluorochrome, such as PI, that is capable of binding and labelling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis and subsequent identification of hypodiploid cells (Riccardi & Nicoletti, 2006). Furthermore, one of the major characteristics of apoptosis is the formation of fragmented DNA, and the determination of the increase in the subG1 population within control and treated cells is representative of the formation of mono- and oligonucleosomes (Darzynkiewicz & Bedner, 2000; Nicoletti et al., 1991). A subG1 peak in the FACS histogram represents the percentage of apoptotic cells with a DNA content of less than 2n (subG1 DNA).

According to the current understanding of apoptosis, two classes of caspase, initiators and effectors, are involved in its mechanism (Cryns & Yuan, 1998). The signals for apoptosis activate initiator caspases (caspase-2, -8 and -9), and the activated initiator caspases trigger the activation of effector caspases (caspase-3, -6 and -7), which in turn activate endonucleases and cause cell collapse by cleaving specific substrates (Enari et al., 1998). We have previously reported the involvement of caspase-3 and -9 in apoptosis of macrophages infected with A. actinomycetemcomitans (Kasai et al., 2004; Nonaka et al., 2001). When infected J774.1 cells were cultured with 100 μM caspase-9, -3/7 or -6 inhibitor, there was a substantial decrease in the population in the subG1 phase (Fig. 1b). A time-course study indicated that the presence of inhibitors at 100 μM also significantly suppressed apoptotic cell death (Fig. 1c). In apoptotic cells, most of the morphological changes are caused by the active caspase, which is thought to control the central executions of the apoptotic pathway. It is well known that caspase-3, -6 and -7 are downstream effectors in the caspase cascade and they have been implicated in many different apoptotic processes (Chen et al., 1996). These effector caspases act downstream and are involved in the cleavage of specific cellular substrate proteins, leading to morphological and biochemical features characteristic of apoptosis (Thornberry & Lazebnik, 1998). Apoptotic changes also have been found to be triggered by the activation of caspases and activated DNase (CAD/DF440) (Enari et al., 1998; Kato et al., 1995). In the present study, we found evidence showing cleavage of caspase-6 and -7 in...
J774.1 cells infected with *A. actinomycetemcomitans* using an immunoblot analysis. The highest levels of the cleaved forms of caspase-6 and -7 were detected at 18 and 36 h, respectively, after infection (Fig. 2). These findings suggest that activation of caspase-6 and -7, as well as caspase-3 (Nonaka et al., 2001), induces apoptosis in macrophages infected with *A. actinomycetemcomitans*. Interestingly, cell-cycle arrest was induced in infected J774.1 cells at the G1 phase before the induction of apoptosis, and caspase inhibition had no effect on this phenomenon (data not shown). Studies are under way to clarify the mechanism of cell-cycle arrest in J774.1 cells infected with *A. actinomycetemcomitans*.

Although the synthetic inhibitors are relatively selective for each caspase, one or a few caspases seem to recognize and cleave the substrates (Ghayur et al., 1996; Takahashi et al., 1996), and application of these inhibitory reagents is an effective approach for dissecting the signalling pathways of apoptotic cell death. We examined the effect of caspase-9 and -3 inhibitors on the cleavage of caspase-6 and -7. Fig. 3 shows that the caspase-9 inhibitor blocked expression of the cleaved forms of caspase-6 and -7, whilst the caspase-3 inhibitor blocked expression of the cleaved form of caspase-7, but not that of caspase-6.

The cellular morphology associated with the apoptotic process has been well characterized by chromosome condensation, membrane blebbing, formation of apoptotic bodies and DNA fragmentation. These apoptotic changes are the result of the cleavage of cellular proteins, such as lamin A/C and PARP, by a family of caspases (Alnemri et al., 1996; Kaufmann et al., 1993; Zhuang et al., 1998). Lamins are nuclear membrane structural components that are important for maintaining normal cell functions, such as cell-cycle control, DNA replication and chromatin organization (Goldberg et al., 1999; Gruenbaum et al., 2000; Yabuki et al., 1999).

Fig. 1. Effect of peptide caspase inhibitors on apoptosis in *A. actinomycetemcomitans*-infected J774.1 cells. J774.1 cells (2×10⁴) were infected with *A. actinomycetemcomitans* at a bacterium:cell ratio of 5000:1. Apoptotic cells were detected by flow cytometric analysis. After culture for 48 h in the presence of caspase inhibitors, the cells were stained with PI. The subG₁ peak in the FACS histogram represents the percentage of apoptotic cells. Histograms for (a) uninfected (i) and infected (ii) J774.1 cells without caspase inhibitors, and (b) infected J774.1 cells with 100 μM caspase-9 inhibitor (i), caspase-6 inhibitor (ii) or caspase-3/7 inhibitor (iii) are shown. (c) After culture for 36 h (white bars) or 48 h (black bars) in the presence of caspase inhibitors, the percentage inhibition was calculated using the following formula: percentage inhibition = 100 × (amount of hypodiploid DNA with caspase inhibitor/amount of hypodiploid DNA without caspase inhibitor). Data are shown as the mean±SD of triplicate samples. The experiments were performed three times, with similar results obtained in each. There were significant differences between the amount of hypodiploid DNA with and without caspase inhibitor (*P*<0.05), but no significant differences between each group with caspase inhibitors.

Fig. 2. Detection of the cleaved forms of caspase-6 and -7 in *A. actinomycetemcomitans*-infected J774.1 cells. Infected J774.1 cells were cultured for the times indicated. Each sample (20 μg protein) was separated on a 15 or 12.5% polyacrylamide gel containing 0.1% SDS to detect caspase-6 and -7, respectively. Proteins were electroblotted onto PVDF membranes and analysed by immunoblotting as described in Methods. N, Non-infected cells.

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structural proteins of the nuclear envelope, has been observed in different cells undergoing apoptosis (Orth et al., 1996; Takahashi et al., 1996; Zhivotovsky et al., 1997), and lamin A/C is cleaved to a small fragment (28 kDa) during the induction of apoptosis (Goldberg et al., 1999; Orth et al., 1996). PARP appears to be involved in DNA repair, predominantly in response to environmental stress (Satoh & Lindahl, 1992), and is important for cells to maintain their viability, whilst the cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Oliver et al., 1998). In the present study, immunoblot analysis revealed that the cleaved

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**Fig. 3.** Effect of peptide caspase inhibitors on the cleavage of caspase-6 and -7 in *A. actinomycetemcomitans*-infected J774.1 cells. To detect the active forms of caspase-6 and -7, infected J774.1 cells were cultured with or without the caspase-9 and -3 inhibitors (100 μM) for 24 h (caspase-6) or 30 h (caspase-7). Each sample (20 μg protein) was separated on a 15 or 12.5% polyacrylamide gel containing 0.1% SDS to detect caspase-6 and -7, respectively. Proteins were electroblotted onto PVDF membranes and analysed by immunoblotting as described in Methods.

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**Fig. 4.** Detection of cleaved lamin A/C in *A. actinomycetemcomitans*-infected J774.1 cells. Infected cells were cultured for the times indicated. Each sample (20 μg protein) was separated on a 12.5% polyacrylamide gel containing 0.1% SDS to detect the cleaved lamin A/C. Proteins were electroblotted onto PVDF membranes and analysed by immunoblotting as described in Methods. N, Non-infected cells.

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**Fig. 5.** Detection of cleaved PARP in *A. actinomycetemcomitans*-infected J774.1 cells. Infected cells were cultured for the times indicated. Each sample (20 μg protein) was separated on a 7.5% polyacrylamide gel containing 0.1% SDS to detect the cleaved PARP. Proteins were electroblotted onto PVDF membranes and analysed by immunoblotting as described in Methods. N, Non-infected cells.

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**Fig. 6.** Effect of peptide caspase inhibitors on the cleavage of lamin A/C and PARP in *A. actinomycetemcomitans*-infected J774.1 cells. Infected cells were cultured with or without caspase-3, -3/7 or -6 inhibitors (100 μM) for 24 h. Each sample (20 μg protein) was separated on a 12.5 or 7.5% polyacrylamide gel containing 0.1% SDS to detect cleaved lamin A/C (a) or PARP (b), respectively. Proteins were electroblotted onto PVDF membranes and analysed by immunoblotting as described in Methods.
fragment of lamin A(C) (28 kDa) was detected maximally at 30 h after infection (Fig. 4). In addition, the cleaved fragment of PARP (85 kDa) was clearly detected maximally at 36 h after infection, during the final execution phase of apoptosis (Fig. 5).

Next, we attempted to clarify the role of caspases in the degradation of lamin A/C and PARP in A. actinomycetemcomitans-infected J774.1 cells. At 24 h after infection, cleavage of lamin A/C was not detected in cells incubated with the caspase-6 inhibitor, and its cleavage was barely detectable in cells incubated with the caspase-3 and -3/7 inhibitors (Fig. 6a). PARP cleavage was detected in cells incubated with the caspase-3 and -6 inhibitors at 30 h after infection. Interestingly, the expression of the cleaved form of PARP (85 kDa) was decreased in infected cells by the caspase-3/7 inhibitor (Fig. 6b).

In conclusion, we found by flow cytometric analysis that caspase-9, -6 and -3/7 inhibitors blocked the induction of apoptosis in macrophages infected with A. actinomycetemcomitans. We also found that infection with A. actinomycetemcomitans induced apoptosis in macrophages through activated caspase-6, after which cleavage of lamin A/C occurred. In addition, immunoblot analysis revealed cleavage of PARP, whilst caspase-3/7 inhibitor blocked cleavage of PARP, whilst caspase-3 inhibitor blocked cleavage in macrophages infected with A. actinomycetemcomitans. These results indicate that the activated effector caspases, caspase-6 and -7, play a critical role in the degradation of lamin A/C and PARP in apoptotic macrophages infected with A. actinomycetemcomitans. Our results also demonstrate that application of caspase inhibitors may suppress the induction of apoptosis in macrophages infected with periodontopathic bacteria.

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REFERENCES


