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Chlamydia (Chlamydophila) pneumoniae-induced cell death in human coronary artery endothelial cells is caspase-independent and accompanied by subcellular translocations of Bax and apoptosis-inducing factor

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Abstract

Atherosclerosis and coronary heart disease are causing high morbidity and mortality worldwide. Different risk factors have been demonstrated, but the exact mechanisms behind these diseases are still not fully understood. Recent studies have suggested Chlamydia pneumoniae to be involved in the pathogenesis, and increased apoptotic indexes in atherosclerotic plaques have been documented. In this study, we show that C. pneumoniae induces apoptosis and necrosis in populations of human coronary artery endothelial cells. Apoptosis was determined by TUNEL and flow cytometry after staining of cells with annexin V and propidium iodide, and defined as TUNEL-reactive or annexin V-positive, propidium iodide-negative cells. The apoptosis was induced within 2 h postinfection and increased with inoculation dose. The general caspase inhibitor z-VAD-fmk did not affect apoptotic frequencies. By immunochemistry and immunoblot, we demonstrated activation and subcellular translocation of the proapoptotic protein Bax, and translocation of apoptosis-inducing factor from the cytosol to the nucleus. These results indicate that C. pneumoniae-induced apoptosis in human coronary artery endothelial cells is caspase-independent and regulated by Bax and apoptosis-inducing factor.
Introduction

The family Chlamydiaceae comprises three human pathogenic species, Chlamydia (Chlamydomphila) pneumoniae, Chlamydia (Chlamydomphila) psittaci and Chlamydia trachomatis. Chlamydiaceae are obligate intracellular pathogens. The extracellular form, elementary body (EB), is internalized by endocytosis into susceptible host cells, is converted to metabolically active reticulate bodies, replicates inside a membrane-bound inclusion, and finally redifferentiates into EBs that are released from the host cell after 2–3 days (Moulder, 1991). During this growth cycle, Chlamydia-containing endosomes avoid fusion with lysosomes, and the normal trafficking of intracellular vacuoles is interrupted (Majeed et al., 1994; Hackstadt et al., 1996; Scidmore et al., 1996). Chlamydia pneumoniae causes human respiratory tract infections and has recently been implicated in the development of atherosclerosis (Saikku et al., 1988). The process of atherosclerosis is believed to be a response to endothelial damage, resulting in dysregulated proliferation and inflammation, causing increased adhesivity of leukocytes and deposition of oxidized lipids in the vessel wall. A possible involvement of C. pneumoniae has been studied using different strategies.

1 Seroepidemiological studies show that patients with coronary artery disease more frequently display C. pneumoniae antibodies than healthy matched individuals (Saikku et al., 1988; Grayston, 2000).

2 Chlamydia pneumoniae has been isolated from atherosclerotic tissues (Taylor-Robinson & Thomas, 2000). The pathogen is considered to be transported by monocytes from the respiratory system and has been shown to infect a range of different cells in the atherosclerotic plaque, such as human endothelial cells, human macrophages and human smooth muscle cells (Gaydos et al., 1996; Maas et al., 2000). Chlamydia pneumoniae infection of macrophages and endothelial cells stimulates uptake and oxidation of low-density lipoprotein (LDL) and accentuates the transformation of monocytes and macrophages into foam cells (Kalayoglu & Byrne, 1998; Dittrich et al., 2004).

3 Studies in rabbit and mouse models have shown that intravascular and intranasal inoculation with C. pneumoniae affect arterial intimal thickening. This may be prevented by treatment with azithromycin (Moazed et al., 1999; Muhlestein, 2000). However, antichlamydial treatment in humans does not appear to give distinct and long-lasting protection against coronary heart disease (O'Connor et al., 2003).

Apoptotic cell death is frequent in inflammatory disorders of the vessel wall, for example in atherosclerosis (Walsh et al., 2000). In unstable atherosclerotic plaques, apoptosis is elevated. Apoptosis is important during tissue differentiation and remodelling (Bosman et al., 1996; Richter et al., 1996; Hetts, 1998), but when dysregulated it may contribute to various diseases such as AIDS and cancer (Thomas, 1995). The key features of apoptosis are the loss of cell junctions and adhesion, fragmentation of DNA, cell shrinkage, and formation of apoptotic bodies that are phagocytosed by neighbouring cells (Bosman et al., 1996). Apoptosis can be initiated by ligation of so-called death receptors at the cell surface (e.g. tumour necrosis factor-α or Fas) or by intracellular signalling (e.g. oxidative stress). Most apoptotic stimuli activate a family of cysteine proteases, the caspases. This activation leads to a cascade of proteolytic cleavage of downstream effector caspases and a number of vital nuclear proteins, leading to cell death (Nunez et al., 1998).
Mitochondria are believed to be central in the execution of apoptosis, through the release of different apoptogenic proteins (Parone et al., 2002). The release of cytochrome c from mitochondria activates the caspase cascade by binding to the adaptor molecule Apaf-1 (Bossy-Wetzel & Green, 1999). The Bcl-2 protein family regulates the release of cytochrome c and thereby initiates or suppresses apoptosis. The release of another mitochondrial protein, the flavoprotein apoptosis-inducing factor (AIF), triggers apoptosis without involvement of caspases (Susin et al., 1999; Candé et al., 2002b). AIF localises in mitochondria, but translocates to the nucleus when apoptosis is induced (Candé et al., 2002b).

Infectious agents require an intimate relationship between the host cell and the pathogen. This is of special importance for viruses and intracellular bacteria that are dependent on the host cell for replication. To withstand the immunological response and to complete the replication process, many pathogens have evolved strategies for both apoptosis induction and inhibition (Grässmé et al., 2001; Häcker & Fischer, 2002). The different chlamydial species have been shown to display both pro- and antiapoptotic properties. We have previously shown C. trachomatis to induce apoptosis in neighbouring uninfected cells (Schöier et al., 2001). In the case of C. pneumoniae, Goth & Stephens (2001) showed phosphatidylserine to be exposed on the host cell surface during C. pneumoniae infection. Recently, Dumrese et al. (2005) reported what they called aponecrosis in human aortic smooth muscle cells infected with C. pneumoniae. A number of studies have shown C. pneumoniae to inhibit apoptosis, generated by different external apoptosis inducers, or spontaneous, physiological cell death (Geng et al., 2000; Fischer et al., 2001, 2004; Rajalingam et al., 2001; Wahl et al., 2001; Arienne et al., 2002; Carratelli et al., 2002; van Zandbergen et al., 2004). The purpose of this work was to study apoptotic cell death in human coronary artery endothelial cells (HCAECs) during C. pneumoniae infection and to characterize the mechanisms regulating apoptosis induction.

Materials and methods

Reagents

Propidium iodide, staurosporine and Triton X-100 were purchased from Sigma (St Louis, MO). Eagle's minimum essential medium, gentamicin, glutamine, trypsin and nonessential amino acids were obtained from ICN Biomedicals (Costa Mesa, CA). EGM-MV bullet-kit medium, HEPES-buffered saline solution, trypsin/EDTA and trypsin-neutralizing solution were obtained from Clonetics (Walkersville, MD). Vectashield and diaminobenzidine tetrahydrochloride (DAB) were obtained from Vector Laboratories Inc. (Burlingame, CA), and Entellan, haematoxylin and methyl green from Merck (Darmstadt, Germany). The fluorescein isothiocyanate-conjugated chlamydial lipopolysaccharide (LPS) antibodies (Phadebact) were obtained from Boule Diagnostics AB (Huddinge, Sweden), 4% neutral buffered formaldehyde from Histolab (Gothenburg, Sweden) and fetal bovine serum from GIBCO (Gaithersburg, MD). Butanol-1 and xylene were obtained from KEBO AB (Spånga, Sweden). All other chemicals were of analytical grade and obtained from standard sources.
Cells

Two human cell types were used. HEp-2 cells (American Type Culture Collection CCL 23), a continuous human larynx carcinoma cell line, were grown in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, gentamicin (10 mg L$^{-1}$), 2 mM glutamine and 1% nonessential amino acids (growth medium, GM). HCAECs were purchased from Clonetics (Walkersville, MA) and subcultured less than 10 passages in specially supplemented medium (EGM2-MV bullet kit). The cells were grown in culture plates to 70–90% confluency and the medium was substituted every second day.

Chlamydial strains and infectious procedures

The C. pneumoniae isolates IOL-207 and T-45 (the latter from the 'Byskepidemin', Sweden) were propagated in HEp-2 cells for 3 days at 37°C in GM containing 1 mg L$^{-1}$ cycloheximide. Infected cells were detached from the monolayer using a rubber policeman, vortexed in a vial containing glass beads and centrifuged at 900 g for 10 min. After centrifugation of the supernatant at 10 000 g for 30 min, the pellet was suspended in sucrose–phosphate buffer supplemented with gentamicin (10 mg L$^{-1}$), amphotericin B (2.5 mg L$^{-1}$), vancomycin (100 mg L$^{-1}$) and 10% fetal calf serum. The number of bacteria, expressed as inclusion forming units (IFUs) in cycloheximide-treated HEp-2 cells, was calculated before storage at −70°C. Cells and C. pneumoniae stock suspensions were regularly analysed by PCR using broad-range 16S rRNA primers (PBR5'se 5'GAAGAGTTTGATCATGGCTCAG3' and P13B 5'GTGTACTAGGCCCGGAACGTATTTC3') and found to be mycoplasma-free. HCAECs were grown in 6- or 24-well plates were used as host cells to determine induction and regulation of cell death. Indicated multiplicities of infection (MOI) were added to HCAECs prior to centrifugation at 3000 g for 1 h. After incubation for 2 h at 37°C, the medium was discarded and fresh GM without cycloheximide added. The cells were then incubated for indicated times at 37°C in cycloheximide-free GM. The MOI 1.25 and 10, based on IFU titration in cycloheximide-containing HEp-2 cells, correspond to approximately MOI 0.125 and 1, respectively, in cycloheximide-free HCAECs, i.e. during the experimental conditions of apoptosis induction. Thus, growth of C. pneumoniae is much less efficient in cycloheximide-free HCAECs than in cycloheximide-containing HEp-2 cells. Where indicated, cells were treated with the apoptosis inductor staurosporine.

Determination of cell death

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)

Cells were inoculated with MOI 1.25 and 10 of C. pneumoniae for 24, 48 and 72 h. The Apoptag in situ apoptosis detection kit from Oncor (Gaithersburg, MD) was used following the manufacturer's instructions, using a procedure modified from that described by Gavrieli et al. (1992). Briefly, the culture medium was removed and adherent cells were fixed in 4% neutral buffered formaldehyde and 0.5% Triton X-100 for 20 min, before TdT enzyme and nucleotides (dUTPs) tailed with digoxigenin were added for 1 h at 37°C, to bind fragmented
free DNA. Thereafter, antidigoxigenin peroxidase was added for 30 min, to label digoxigenin-dUTP. Apoptotic nuclei were stained by 4 min of incubation in the peroxidase substrate DAB, before counterstaining with haematoxylin. After dehydration with butanol and xylen, cells were mounted in Entellan. TUNEL-positive cells were identified blindly by light microscopy at × 400 magnification in approximately five randomly selected fields, yielding a total number of at least 200 cells in each sample. Cell shrinkage combined with DAB positivity was required to classify a cell as apoptotic. As positive control, cells were treated with 1 μM staurosporine for 2 or 24 h prior to TUNEL staining. In negative controls, TdT-enzyme and dUTPs were excluded.

Flow cytometry

Flow cytometry was used to determine cell death by two assays: propidium iodide (PI) staining of methanol-fixed cells, and annexin V+PI staining of unfixed cells. In the first assay, cells were inoculated with MOI 1.25 and 10 of C. pneumoniae for 12, 24, 48 or 72 h. The procedure was performed with smaller modifications compared with that described by Nicoletti et al. (1991). After infection of HCAECs with C. pneumoniae for the indicated times, the growth medium from each well, containing released cells, was collected and the adherent cells were detached by trypsination and added to the medium. After centrifugation at 1000 g for 10 min, the pelleted cells were fixed in 70% methanol for 5 min at room temperature before centrifugation at 5000 g for 35 s. The supernatant was then removed, and PI (at a final concentration of 6.3 μM) was added to the pellet for 5 min at room temperature to stain the chromatin. The cells were finally processed in a Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA). PI intensity was evaluated from at least 8000 cells in each sample, using the data program Cell Quest. To discriminate apoptosis from cytotoxicity, the analysis was performed after PI staining of unfixed infected cells and of cells treated with staurosporine, and fluorescence intensity gated according to our previous protocol (Schöier et al., 2001). In the assay combining annexin V and PI, HCAECs were infected with C. pneumoniae T45 MOI 0.1–50 and incubated for 2–72 h. The culture medium was then decanted, and 500 μL of annexin V (Annexin V Fluos, Roche Diagnostics, Penzberg, Germany) and PI were added for 10 min according to the manufacturer's instructions. Cells were rinsed and detached with a rubber policeman, centrifuged at 500 g for 5 min and resuspended in the incubation buffer supplied in the kit. Annexin V and PI intensities were evaluated from 10 000 cells in the FACS Calibur using Cell Quest. Annexin V-positive and PI-negative cells were considered apoptotic, and PI-positive cells were considered necrotic.

Inhibition of caspases

Human coronary artery endothelial cells were preincubated for 60 min with the general caspase inhibitor z-VAD-fmk (Pharmingen, San Diego, CA) before addition of C. pneumoniae T45 (MOI 1.25 or 10). The inhibitor was diluted in dimethylsulphoxide (DMSO) (final concentration 0.2%) and added to the medium at a concentration of 50 μM. Apoptosis was determined in the presence or absence of inhibitor by TUNEL after 48 h of incubation at 37°C. Cells treated with 1 μM staurosporine for 4 h were used as a control for caspase-dependent apoptosis. The influence of solvents, ethanol or DMSO on TUNEL reactivity was evaluated.
**Immunofluorescence microscopy**

Human coronary artery endothelial cells were grown on coverslips to 70–90% confluency, and infected with C. pneumoniae at indicated MOI and for indicated incubation times. Cells were washed twice in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 15 min. After washing, cells were incubated for 30 min in N-20 anti-Bax rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 : 50 dilution in permeabilization buffer (bovine serum albumin (BSA) 1 g L$^{-1}$, 0.05% saponin in PBS), washed twice in PBS-BSA, blocked in 4% normal goat serum for 30 min, washed in PBS-BSA and visualized by incubation for 30 min with a 1 : 100 dilution of Alexa Fluor 568 F(ab')2 goat antirabbit IgG antibody (Molecular Probes, Eugene, OR). To visualize AIF, cells were incubated with goat polyclonal antibody (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 : 25 dilution, blocked in 2% BSA and visualized after incubation with a 1 : 50 dilution of Alexa Fluor 488 rabbit antigoat antibody (Molecular Probes, Eugene, OR). Samples were mounted in Vectashield and viewed with a Leitz Diaplan microscope (Leica Microsystems Limited, Cambridge, UK) and analysed by Adobe Photoshop software.

**Immunoblot**

Chlamydia pneumoniae T45-infected HCAECs were detached from 6-well plates, centrifuged at 600 g for 10 min, rinsed in PBS and resuspended in buffer A (20 mM Hepes, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride and 250 mM sucrose, pH 7.5) (Wu et al., 2002). Cells were homogenized for about 100 strokes with a Dounce homogenizer, and centrifuged at 1000 g for 10 min at 4°C; the pellet, enriched in nuclei and unbroken cells, was resuspended in buffer A. The supernatant was spun at 10 000 g for 60 min at 4°C, and the resulting pellet and supernatant (cytosolic fraction) was stored at −70°C. Selected samples were lysed in a buffer containing 62.5 mM Tris-HCl, 2% sodium dodecylsulphate (SDS) and 10% glycerol. Equal amounts of protein were subjected to 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Hybond PVDF membranes, and blocked in 5% ECL blocking agent containing 3% BSA overnight. The membrane was incubated with anti-Bax antibody (N-20) diluted 1 : 2000 in PBS–Tween-20 – enhanced chemiluminescence blocking agent or with anti-AIF (N-19) antibody diluted 1 : 400 (Santa Cruz Biotechnology, Santa Cruz, CA) for 60–90 min, washed and treated with horseradish peroxidase-conjugated antibody at 1 : 2000 dilution (Amersham Biosciences, Uppsala, Sweden; Dako, Glostrup, Denmark) for 60 min, incubated with streptavidin HRP, and visualized with the ECL Plus WB detection system.
Statistics

Data on apoptotic frequencies were expressed as mean and standard deviation (SD). The statistical analysis was carried out using anova.

Results

Morphological characteristics of Chlamydia pneumoniae infection in HCAECs

In the Materials and methods section, it is mentioned that MOI of 1.25 and 10 for cycloheximide-treated HEp-2 cells correspond to approximately MOI of 0.125 and 1 in cycloheximide-free HCAECs. These MOI are based on the average IFU titer of five to seven 10-fold dilutions of C. pneumoniae in the two cell types. The results suggest that the development of inclusions was about 10 times less efficient in HCAECs than in HEp-2 cells.

To explore the infectivity further, HCAECs were inoculated with MOI 0.1–50, incubated for 72 h and stained with the LPS antibodies. These MOI were calculated from IFU titration in HEp-2 cells. Two morphological, immunoreactive forms of C. pneumoniae were observed associated with HCAECs; round to oval intracellular inclusions and spots/aggregates. Individual cells could contain single or multiple inclusions. The calculated MOI used in this study are based on number of developed inclusions. Cells with spots/aggregates but without inclusions were not included in the IFU titre determination. As expected, the number of inclusions and spots/aggregates increased with inoculum size. However, even at MOI 50, all cells did not contain fully developed inclusions. In detail, at MOI 50, 90–100% of cells contained spots/aggregates and 5.5% contained inclusions. The corresponding values for MOI 10, 1 and 0.1 were 50% and 3.6%, 20–30% and 1.7%, and 5–10% and 0.12%, respectively. Thus, the ratio spots/aggregates to inclusions varies from 10 to 80 for the inocula used.

Chlamydia pneumoniae induces apoptosis in HCAECs

Human coronary artery endothelial cells were inoculated with C. pneumoniae isolates T45 and IOL-207 and cell death was determined after 2–72 h at MOI 0.1–50. To quantify cell death, flow cytometry and the TUNEL method were used.

Apoptotic cells are smaller than normal cells and exhibit nuclear condensation. They can, therefore, after fixation and PI staining, be quantitatively detected as a hypodiploid cell population in flow cytometric analysis (Nicoletti et al., 1991).

After 12 h of incubation with C. pneumoniae isolate T45, a significant (P<0.05) increase in PI-positive, methanol-fixed cells was observed by flow cytometry for both MOI 1.25 and MOI 10 compared with uninfected controls (Fig 1a). Corresponding increases in PI-positive cells occurred in infected cells after 24, 48 and 72 h of incubation for all isolate–inocula combinations, except after 24 h of incubation with T45 at MOI 1.25, where the values did not reach statistical significance compared with uninfected controls (Fig 1a).
Fig. 1. Apoptotic indexes of human coronary artery endothelial cells as determined by (a) flow cytometry of propidium iodide-stained, methanol-fixed cells, or (b) terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling. Cells were infected with different multiplicities of infection of Chlamydia pneumoniae T45 or IOL-207 for 12, 24, 48 or 72 h. Values represent mean±standard deviation of two to 10 experiments. *P<0.05.

In the TUNEL assay, DAB-stained cells containing a condensed nucleus were considered apoptotic. This assay confirmed the flow cytometry data, showing a significant (P<0.05) increase in percentage of apoptotic cells for all combinations of C. pneumoniae isolates and inocula after 24, 48 and 72 h, except for isolate IOL-207, MOI 1.25 (Fig 1b). Furthermore, a significant (P<0.05) increase in TUNEL-positive cells was observed in cells infected with T45 MOI 10 compared with MOI 1.25 for all four incubation times by flow cytometry of fixed PI-stained cells and TUNEL. A corresponding dose dependency was not observed for C. pneumoniae IOL-207. The TUNEL assay also showed significantly (P<0.05) higher levels of apoptosis in cells infected with T45 MOI 10 at 24 and 48 h compared with cells infected with isolate IOL-207 MOI 10. A corresponding difference between the two isolates was also revealed by flow cytometry after 24 h of incubation (Fig. 1a and b).
Flow cytometry of unfixed cells stained with both annexin V and PI confirmed the above findings. Annexin V fluorescence intensity increased more than the PI intensity when uninfected HCAECs and HCAECs infected with MOI 10 for 24 h were compared (Fig. 2). When apoptosis was defined as annexin V-positive, PI-negative cells, apoptosis was evident as early as 2 h postinoculation. The number of apoptotic cells increased with inocula and incubation times (Table 1). In cells infected with MOI 50 for 48 h, the increase of apoptotic cells was 125% compared with uninfected cells. These results were obtained with adherent cells after removal of cells detached from the wells during incubation. When these detached cells were analysed separately from wells with infected cells that were incubated for 48 h or more, 95–100% of annexin V-positive cells were also PI positive; that is, these detached cells were necrotic, whereas a population of adherent cells were apoptotic. Furthermore, the number of PI-positive cells, representing a necrotic cell population, also increased in adherent cells after infection with C. pneumoniae T45. This is in contrast to cells treated with staurosporine, where the number of annexin V-positive but not the number of PI-positive cells increased compared with untreated cells.

Taken together, these results show that C. pneumoniae induces both apoptosis and necrosis in HCAECs. The apoptosis starts within 2 h postinoculation and is dose-dependent for at least one of the tested C. pneumoniae isolates.
Fig. 2. Apoptotic and necrotic cell populations in (a) uninfected human coronary artery endothelial cells (HCAECs) and (b) HCAECs infected with Chlamydia pneumoniae T45 at multiplicity of infection 10 for 24 h. Adherent cells were stained with annexin V (thin lines) and propidium iodide (thick lines), detached, and analysed by flow cytometry.
Table 1. Apoptotic and necrotic cell populations in human coronary artery endothelial cells (HCAECs) infected with Chlamydia pneumoniae T45

<table>
<thead>
<tr>
<th></th>
<th>Annexin V⁺ PI⁺</th>
<th>Annexin V⁺/PI⁻</th>
<th>Percentage increase of Annexin V⁺/PI⁻ compared with uninfected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected; 2 h</td>
<td>3271</td>
<td>954</td>
<td>2317</td>
</tr>
<tr>
<td>MOI 0.1; 2 h</td>
<td>3192</td>
<td>933</td>
<td>2259</td>
</tr>
<tr>
<td>MOI 1; 2 h</td>
<td>3689</td>
<td>1105</td>
<td>2584</td>
</tr>
<tr>
<td>MOI 10; 2 h</td>
<td>3517</td>
<td>940</td>
<td>2577</td>
</tr>
<tr>
<td>MOI 50; 2 h</td>
<td>4331</td>
<td>1080</td>
<td>3251</td>
</tr>
<tr>
<td>Uninfected; 24 h</td>
<td>2138</td>
<td>554</td>
<td>1584</td>
</tr>
<tr>
<td>MOI 1; 24 h</td>
<td>2739</td>
<td>918</td>
<td>1821</td>
</tr>
<tr>
<td>MOI 10; 24 h</td>
<td>3480</td>
<td>1309</td>
<td>2171</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>3911</td>
<td>580</td>
<td>3331</td>
</tr>
<tr>
<td>1 μM; 24 h</td>
<td></td>
<td></td>
<td>110</td>
</tr>
</tbody>
</table>

Table shows number of cells from representative experiments with HCAECs infected at multiplicities of infection (MOI) 0.1–50 for 2 and 24 h. Adherent cells were stained with annexin V and propidium iodide (PI), detached and analysed by flow cytometry. Annexin V-positive and PI-negative cells were considered apoptotic, and PI-positive cells necrotic.

HCAECs, human coronary artery endothelial cells; NA, not applicable.

**Chlamydia pneumoniae-induced apoptosis in HCAECs is caspase-independent**

Human coronary artery endothelial cells were infected with C. pneumoniae isolate T45 at MOI 1.25 or 10 for 48 h with or without the general caspase inhibitor z-VAD-fmk. Apoptosis was measured with the TUNEL assay. The presence of inhibitor did not significantly change the percentage of apoptotic cells in comparison with infected, inhibitor-free cell preparations (Fig. 3). As a control, 50 μM z-VAD-fmk was demonstrated to block staurosporine-induced apoptosis in HCAECs.
Lack of effect of caspase inhibitor on Chlamydia-induced cell death. Apoptotic indexes of human coronary artery endothelial cells as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling. Cells were infected with different multiplicities of infection of C. pneumoniae T45 for 48 h or treated with 1 μM staurosporine for 4 h in the presence or absence of the general caspase inhibitor z-VAD-fmk. Values from infected and uninfected cells represent mean±standard deviation of five or six experiments; values from staurosporine controls are from one or two experiments.

Subcellular redistribution of Bax and AIF in Chlamydia pneumoniae-infected HCAECs

Bax is a proapoptotic member of the Bcl-2 protein family. During apoptosis, Bax is activated and translocates from the cytosol to mitochondria, where it regulates release of cytochrome c (Wolter et al., 1997; Desagher et al., 1999). To investigate whether Bax is involved in C. pneumoniae-induced cell death, the subcellular distribution of Bax was determined by immunofluorescence. Three to four per cent of C. pneumoniae-infected cells displayed a punctate pattern of fluorescence after 8 h of incubation compared with 1.5% in mock-infected cells (P<0.05) (Fig. 4a). The other cells showed diffuse cytoplasmic staining with the anti-Bax antibody (Fig. 4 b and c). Also, after 24 h of incubation, there was a statistically significant increase in cells with punctate fluorescence in infected monolayers (MOI 1.25) compared with uninfected monolayers (data not shown).
Fig. 4. Subcellular translocation of Bax during Chlamydia pneumoniae infection of human coronary artery endothelial cells. (a) Cells were infected with Chlamydia pneumoniae T45 at multiplicities of infection 1.25 or 10 for 8 h and incubated with N-20 polyclonal antibody towards Bax and secondary antibody. Cells with a punctate fluorescence pattern were enumerated by fluorescence microscopy. Bars represent mean±standard deviation of six or seven experiments. *P<0.05. (b) Subcellular localization of Bax in uninfected cells showing diffuse cytosolic fluorescence. (c) Subcellular localization of Bax in cells infected with C. pneumoniae, multiplicity of infection 1.25, for 24 h showing punctate cytosolic fluorescence.
Apoptosis-inducing factor is a mitochondrial intermembrane flavoprotein with homology to bacterial oxidoreductases (Susin et al., 1999). AIF is normally confined to mitochondria and participates in regulation of apoptosis via a caspase-independent pathway. Specific apoptotic signals received by a cell trigger the opening of mitochondrial permeability transition pores and allow AIF to be released from mitochondria into cytosol and subsequently translocated to the nucleus. To determine a possible involvement of AIF in C. pneumoniae-induced apoptosis, infected cells were stained with anti-AIF antibody and analysed for translocation from mitochondria to the nucleus. After 8 h of incubation with C. pneumoniae (MOI 10), cells with nuclear fluorescence had already increased to 8–9% compared with about 4% in uninfected cells (P<0.05) (Fig. 5a). Other cells showed punctate, cytoplasmic staining with the anti-AIF antibody (Fig. 5b and c). Also, after 24 h of incubation, there was a statistically significant increase in cells with nuclear fluorescence in infected monolayers compared with uninfected monolayers (data not shown).

Immunoreactivity towards Bax and AIF in homogenized, fractionated HCAECs was analysed by Western blot. In the post-1000 g pellet, enriched for nuclei and unbroken cells, expression of Bax increased in cells infected with MOI 1 for 6 h. A similar increase was found in staurosporine-treated cells (Fig. 6). A corresponding increase in Bax expression occurred in cells infected with MOI 1 for 3 h, but not in cells infected with MOI 10 or after 24 h of incubation, or in the post-10 000 g pellet. However, Bax expression decreased in the cytosolic post-10 000 g supernatant (Fig. 6). AIF expression increased in the post-1000 g pellet in about half of the experiments where cells were infected with MOI 1 and 10 for 6 or 24 h (Fig. 6). These immunoblot data support the immunochemical findings that Bax and AIF translocate in HCAECs during C. pneumoniae infection.
**Fig. 5.** Subcellular translocation of apoptosis-inducing factor (AIF) during Chlamydia pneumoniae infection of human coronary artery endothelial cells. (a) Cells were infected with Chlamydia pneumoniae T45 at multiplicities of infection 1.25 or 10 for 8 h and incubated with N-19 polyclonal antibody towards AIF and secondary antibody. Cells with nuclear fluorescence were enumerated by fluorescence microscopy. Bars represent mean±standard deviation of nine to 11 experiments. *P<0.05. (b) Subcellular localization of AIF in uninfected cells showing cytosolic fluorescence. (c) Subcellular localization of AIF in cells infected with C. pneumoniae, multiplicity of infection 1.25, for 24 h showing nuclear fluorescence.
Fig. 6. Expression of Bax and apoptosis-inducing factor (AIF) in homogenized, fractionated human coronary artery endothelial cells by immunoblot. AIF: cells incubated for 24 h and the post-1000 g pellet analysed. Bax (n): as for AIF, but cells were incubated for 6 h. Bax (c): cells incubated for 48 h and the post-10 000 g supernatant analysed. 1. Uninfected cells. 2. Cells infected with multiplicity of infection 1. 3. Cells infected with multiplicities of infection 10 and 4. Cells treated with 2 μM staurosporine for 4 h.

Discussion

In this study, we show that a population of Chlamydia pneumoniae-infected HCAECs display apoptotic features, and that a population is also necrotic. HCAECs are highly relevant cells as a model to explore the potential involvement of C. pneumoniae in coronary heart disease. Previous studies have, to a large extent, relied on transformed, established cell lines. A productive infection with development of inclusions occurs in HCAECs, but is only about 10% as efficient as in HEp-2 cells. The majority of HCAECs that are exposed to C. pneumoniae in the model used do not develop morphologically typical inclusions, but instead display spots/aggregates that are immunoreactive with antibodies to Chlamydia. Dumrese et al. (2005) made similar observations for C. pneumoniae infection in human aortic smooth muscle cells, and cell death induction was never found in inclusion-containing cells. The relative efficiency of inclusion development in HCAECs seems to decrease with increasing inocula, since at high MOI the expected number of inclusions do not develop. Apoptosis was determined by TUNEL and flow cytometry after staining of cells with annexin V and PI. Cells reactive in the TUNEL assay and displaying condensed nuclei or cells that were annexin V positive but PI negative were considered apoptotic. The number of apoptotic cells identified in the three assays used varied to some extent. Part of this variation is to be expected, since the assays are based on different principles and criteria for apoptosis. Furthermore, TUNEL and flow cytometry of double-stained cells were analysed on adherent cells, whereas PI staining of fixed cells included both adherent cells and cells detached during the infection period. Phosphatidylinerine exposure as a sign of apoptosis (Jungas et al., 2004) was detected after 2 h of infection. Morphological signs of apoptosis in the form of nuclear condensation were seen after 12 h, and apoptosis determined as TUNEL-positive cells was not affected by a general caspase inhibitor. Apoptosis was also dose-dependent, and apoptotic frequencies were higher in cells infected with a clinical isolate of C. pneumoniae with a limited number of in vitro passages compared with a laboratory-adapted strain.

During apoptosis, several proteins are activated and mobilized to or from the mitochondria. Cytochrome c is a key protein released from mitochondria that regulates downstream apoptotic events (Bossy-Wetzel & Green, 1999). Bax and AIF regulate the release of cytochrome c during apoptosis (Pastorino et al., 1998; Susin et al., 1999). Our finding that Bax is activated and translocated from a diffuse cytosolic distribution in uninfected monolayers to a punctate fluorescence corresponding to mitochondrial distribution in infected
monolayers indicates its role in the regulation of Chlamydia-induced apoptosis. A similar Bax activation has previously been reported in caspase-independent apoptosis in C. psittaci-infected cells (Perfettini et al., 2002). Increased Bax expression in a nuclei- and unbroken cell-enriched fraction of infected cells, and decreased expression in a cytosolic fraction, support subcellular Bax translocation during C. pneumoniae-induced cell death, although we could not verify increased expression of Bax in a heavy membrane- and mitochondria-enriched fraction by immunoblot.

To our knowledge, this study is the first to report the involvement of AIF in C. pneumoniae-induced cell death in HCAECs. During infection, AIF translocates from a cytosolic punctate distribution to a nuclear distribution, and increased expression of AIF occurs in a nuclei- and unbroken cell-enriched fraction of infected cells. This suggests that AIF binding to nuclear components provokes caspase-independent chromatin condensation. Furthermore, AIF can trigger release of cytochrome c from isolated mitochondria (Susin et al., 1999). Crosstalk between AIF and the caspase cascade at several levels and AIF activation secondary to caspase activation have been described (Candé et al., 2002a; Lassus et al., 2002).

How do our findings relate to the mechanisms behind the association between C. pneumoniae infection and coronary heart disease? Endothelial C. pneumoniae infection promotes oxidation of LDL and induces macrophage foam cell formation (Kalayoglu & Byrne, 1998; Dittrich et al., 2004). Oxidized LDL stimulates AIF-regulated apoptosis in endothelial cells (Zhang et al., 2004), and ruptured atheromatous plaques display enhanced apoptosis (Walsh et al., 2000). Recently, it has been shown that C. pneumoniae augments oxidized LDL-induced cell death of mouse macrophages by a caspase-independent pathway (Yaraei et al., 2005). Our study indicates AIF to be a regulator in C. pneumoniae-induced apoptotic cell death. Thus, C. pneumoniae may directly promote apoptosis in coronary cells by AIF and Bax mobilization, or indirectly stimulate apoptosis by oxidation of LDL.

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