**Streptococcus pneumoniae** R6x induced p38 MAPK and JNK-mediated Caspase-dependent apoptosis in human endothelial cells

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**Summary**

*Streptococcus pneumoniae* is the major pathogen of community-acquired pneumonia and a common cause of otitis, meningitis and sepsis. During pneumococcal infection accompanied by bacterial invasion and hemogenous spreading, the endothelium is directly targeted by pneumococci and their virulence factors. Therefore, we tested the hypothesis that pneumococci induced endothelial apoptosis. Unencapsulated R6x pneumococci strongly induced apoptosis of human endothelial cells both from lung microvasculature and umbilical vein, whereas an encapsulated strain D39 mainly led to necrotic cell death. Deletion of the gene coding for pneumolysin reduced pneumococci-induced apoptosis in HUVEC. Furthermore, N-acetyl-L-cysteine, an antioxidant thiol, significantly reduced apoptosis caused by R6x, and LDH release induced by D39, pointing to a role for reactive oxygen species in the pathogenesis. Apoptotic cells showed increased cleavage and activity of caspases 6 and 9 but only late activation of caspase 3. Programmed cell death could be strongly reduced by pan-caspase inhibitor ZVAD. Reduced levels of Bcl2 and cytosolic increase of apoptotic-inducing factor in pneumococci-infected cells implicated involvement of mitochondrial death pathways. Caspase activation and apoptosis were abolished by cAMP elevation. Moreover, p38 mitogen-activated protein kinase and c-Jun NH$_2$-terminal kinase were activated in pneumococci-infected cells and inhibitors of both kinases strongly reduced pneumococci-induced caspase activation and apoptosis. Hence, kinase- and caspase-dependence of pneumococci-induced endothelial apoptosis may bear relevance to novel therapeutic approaches to pneumococci-related disease.

**Keywords**

Endothelium, pneumococci, apoptosis, MAP kinase, caspase

**Introduction**

*Streptococcus pneumoniae* is a very common pathogen in industrialized countries, causing many hospital admissions due to, e.g. pneumococcal pneumonia, meningitis, and sepsis, resulting in high costs for public health systems (1). Pneumococci contain an array of important virulence factors including capsular polysaccharide, as well as $	ext{H}_2	ext{O}_2$ and pneumolysin formation (2). They invade host tissue and target cells, and hemogenous spreading is regularly observed in pneumococci infection (1).

During hemogenous spreading, in sepsis or severe pneumonia, the endothelium is directly exposed to these bacteria and their virulence factors, and endothelial activation by pathogens may significantly influence disease process (3). Recent studies using brain cells (2, 4), leucocytes (5) and lung epithelium (6) demonstrated that pneumococci and pneumococcal cell wall preparations induced programmed cell death by different and specific mechanisms. Although endothelial cells are primary targets of pneumococci, their effect on endothelial viability is widely unknown.

A plethora of different stimuli activate the pro-apoptotic machinery which consists of caspases as central executioners of programmed cell death. The regulatory caspase 8 is directly activated by death-receptors, while caspase 9-activation follows mitochondrial stress (7, 8). Both pathways merge by activating executioner caspases 3 or 6. Different pro-apoptotic (e.g. Bax,
Bid) and anti-apoptotic (e.g. Bcl-2, Bcl-xL) mitochondrial proteins participate in the regulation of apoptosis (8). Also, the mitochondrial apoptosis inducing factor (AIF) has been shown to particularly regulate apoptosis, including pneumococci-related apoptosis (4).

Mitogen-activated protein kinases (MAPK) play a central role in signaling pathways regulating cell proliferation, differentiation, apoptosis and survival (9). Two main players are the stress-induced p38 MAPK and the cJun-NH$_2$-terminal kinase (JNK). Activation of these kinases seem to contribute to bacteria-induced apoptosis (10, 11).

In this study, we tested the hypothesis that S. pneumoniae induced apoptosis of human endothelial cells. Unencapsulated pneumococci R6x induced dose- and time-dependent endothelial apoptosis whereas encapsulated D39 induced endothelial necrosis. Programmed cell death induced by R6x was executed by the mitochondrial pathway, as indicated by reduced Bcl-2 expression, release of AIF, and activation of caspase 6 and 9 in pneumococci-infected endothelium. Both apoptosis and caspase-activation could be blocked by cAMP-elevation. N-acetyl-L-cysteine (NAC), an antioxidant thiol, reduced R6x-related DNA fragmentation and D39-induced LDH-release implicating H$_2$O$_2$-generation in both, apoptosis and necrosis. Moreover, pneumococci activated p38 MAPK and JNK, and inhibition of this pathways reduced programmed cell death as well as activation of caspasess 6 and 9. Taken together, endothelial apoptosis and necrosis may contribute significantly to pneumococci-related disease.

Materials and methods

Materials

ECBM (Endothelial Cell Basal Medium) was provided by PAA, Austria. FCS, trypsin-EDTA-solution and antibiotics were obtained from Life Technologies (Karlsruhe, Germany). Protease inhibitors, Triton X-100, 4-dichloroisocumarin, PMSF, gelatine from porcine skin, staurosporine and Tween-20 were purchased from Sigma Chem. Co. (Munich, Germany). zVAD, SB202190, SB202474 and SP600125 were purchased from Calbiochem (Merck, Darmstadt, Germany). All other chemicals used were of analytical grade and obtained from commercial sources.

Cell culture and infection of HUVEC and HPMEC

Pulmonary endothelial cell line HPMEC-ST1.6R (HPMEC) was a kind gift from Dr. Kirkpatrick, Institute of Pathology, Johannes Gutenberg University, Mainz, Germany (12, 13). HPME cells were grown in HPMEC medium containing M199, 20% FCS, 2 mM Glutamax I, 100 U/100 µg/ml penicillin/streptomycin (Invitrogen Life Technologies, Karlsruhe, Germany), 50 µg/ml ECGS (Becton Dickinson), and seeded into 10 cm dishes, 6- or 96 well plates. Confluent monolayers of HUVEC and HPMEC cultures were used.

A day before infection, cells were supplied with infection medium without antibiotics containing M199 with 1% FCS for HPMEC and ECBM with 1% FCS for HUVEC. Endothelial cells were infected with 10$^5$, 10$^6$ or 10$^7$ bacteria per ml and incubated in infection medium for a given time at 37°C and 5% CO$_2$. Bacteria growth impairment by the medium has not been observed during the experiments.

Bacterial strains

S. pneumoniae R6x is the unencapsulated derivative of serotype 2 strain D39 (18). A pneumolysin-negative mutant of R6x (R6xΔply) was generated by insertion-duplication mutagenesis using the pJD9;ply construct (19). After transformation of the plasmid into S. pneumoniae strain R6x, erythromycin-resistant transformants were selected with Luria-Bertani agar supplemented with erythromycin and 5% sheep blood. PCR analysis and DNA sequencing confirmed insertion of the plasmid into the pneumolysin encoding gene ply. Loss of function was analyzed using the hemolysis assay as described previously (20). Single colony isolates of R6x and its corresponding mutant R6xΔply were maintained at 37°C with 5% CO$_2$ on Columbia agar with 5% sheep blood at 37°C. For cell culture stimulation studies, single colonies were expanded by resuspension in Todd-Hewitt broth supplemented with 0.5% yeast extract and incubated at 37°C for 3–4 h to midlog phase (A$_{600}$ 0.2 – 0.4), harvested by centrifugation and resuspended in the above described infection medium.

Immunofluorescence

1 x 10$^5$ cells grown on glass cover slips were stimulated as indicated, washed twice, and incubated in a humidified atmosphere. Briefly, cells were fixed in freshly prepared paraformaldehyde (3% in PBS, pH 7.6), permeabilized, washed, and DNA strand breaks were labeled by fluorescein (FITC)-dUTP-nick-end-labeling (TUNEL), enhanced by application of Alexa 488 anti FITC antibody (1:1000, 1 h at 37°C), and analyzed by using a Zeiss Pascal 5 confocal microscope. F-actin was visualized by marking with Alexa 546-labeled phalloidin (1:200, 30 min) as described previously (6, 17).

Annexin-V-propidium iodide (PI)-stain

A commercially available Annexin-V-FLUOS staining Kit based on detection of cell-surface phosphatidylserine as marker for apoptotic cells and the DNA-stain propidium iodide for necrotic cells was used (Roche, Mannheim, Germany). 1 x 10$^5$ cells grown on glass cover slips were stimulated as indicated, washed twice, incubated in a humidified atmosphere. Cells were simultaneously stained with Annexin-V-FLUOS (green) and propidium iodide (red) as described previously (17) and visualized by fluorescence microscopy.

Cell death detection ELISA

A commercially available photometric ELISA was used for the detection of cytoplasmic histone-associated-DNA-fragments
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Figure 1: Time- and dose-dependent DNA fragmentation of pneumococci-infected HUVEC. A, HUVEC were infected for indicated times with S. pneumoniae R6x (10^3/10^6 cfu/ml) and DNA fragmentation was measured. * p < 0.05 vs. unstimulated control at single time points. B, HUVEC were incubated with solvent (control), 10^6 cfu/ml and 10^8 cfu/ml S. pneumoniae R6x, or 1 µM staurosporine for 16 h, respectively. DNA fragmentation was detected by TUNEL stain and visualized by confocal laser microscopy. Representatives of three different experiments with similar results were shown. C, HUVEC were incubated with S. pneumoniae R6x or D39 (10^3 cfu/ml, both) for 16 h, or staurosporine (St, 1 µM) for 8 h, respectively. Cells were simultaneously stained with Annexin-V-FLUOS (green) and propidium iodide (red) and visualized by fluorescence microscopy. Representatives of three independent experiments with similar results were shown.

(mono- and oligonucleosomes) in apoptotic endothelial cells (Roche, Mannheim, Germany). 5 x 10^4 cells cultured in 96-well plates were washed twice, incubated in a humidified atmosphere, and stimulated as indicated. Microtiter plates were then centrifuged, medium was removed, cells were lysed, and plates centrifuged again. 20 µl of the supernatant was transferred into a streptavidin-precoated microtiter plate, and incubated with the immunoreagent (anti-histone-biotin, anti-DNA-peroxidase) for 2 h at room temperature. After washing substrate solution was added and absorbance determined at 405 nm (6, 17). Result was expressed as “fold of control”, i.e. the optical density of the solvent treated control was set directly one.

Caspase activity
A commercially available caspase activity assay (ApoTarget, Biosource, Camarillo, CA) based on fluorometric detection of the cleavage of 7-amino-4-trifluoromethyl coumarin-labeled substrate specific for caspase 3 (DEVD), 6 (VEID), or 9 (LEHD) was used for analysis of caspase activity according to manufacturers instructions. Briefly, cells were stimulated as indicated, collected, and lysed on ice. Cleared samples were aligned for protein content, split up into three aliquots and incubated at 37°C for 2 h in presence of labeled caspase-specific fluorometric substrate conjugate for caspase 3, 6, or 9, respectively. Detection was performed by measuring excitation at 390 nm and emission at 510 nm using a fluorometer (Fluoro-Max-2; ISA, Grasbrunn, Germany) (17).

Western blotting
Endothelial cell monolayers grown in 10 cm dishes (5 x 10^6 cells per dish) were stimulated as indicated. Cells were then collected after trypsination, washed, and lysed for 10 min on ice in 20 mM HEPES-buffer, pH 7.4, containing 1 % triton X-100, 44 µg/ml PMSF, 2 µg/ml leupeptin and 2 µg/ml pepstatin. Samples were resuspended in gel-loading buffer according to Laemmli (21), and boiled for 5 min. 60 µg protein per lane were separated on a 10% SDS-PAGE and blotted on Hybond-ECL membranes (Amersham, Dreieich, Germany). Membranes were blocked, washed and hybridized with polyclonal antibodies raised against cleaved caspase 6 (Cell Signaling, Beverly, MA), pro-caspase 3, pro-caspase 9, full length Bax, Bcl2 (Upstate Biotechnology, Lake Placid, NY), or ERK2 (Santa Cruz Biotechnology, Heidelberg, Germany). Detection was performed by visualization of IRDye 800– or Cy5.5-labeled secondary antibodies (Odyssey infrared imaging system, LI-COR Inc., Lincoln, NE). In all experiments, ERK2 was detected simultaneously to check for equal protein load (6, 14, 22, 23).

Release of lactate dehydrogenase (LDH)
HUVEC and HPMEC monolayers were exposed to stimuli as indicated. LDH-activity in the supernatant was determined by the colorimetric measurement of the reduction of sodium pyruvate in the presence of NADH (LDH Assay, Roche, Mannheim, Germany) as described (17). Maximum lysis was induced by adding 100 µl of media containing 0.1% Triton X-100. Percent specific lysis was calculated using the following formula: % specific LDH release = (experimental release – minimum release)/[maximal release – minimum release]) x 100 (6, 24).
this assay, color development is directly proportional to the LDH content (manufacturer information). To address linearity of this assay in the relevant optical density range for our study, we lysed different numbers of human umbilical vein cells (HUVEC) and assessed total LDH activity in additional experiments. We found a linear relationship between the number of lysed cells and the color development (data not shown).

**Statistical methods**

Data are shown as means ± SEM of at least three independent experiments. A one-way ANOVA was used for data of figure 1A, 2, 3A/C-F, 5 and 6B-D (single time points). Main effects were then compared by a Newman-Keuls’ post-test. p<0.05 was considered to be significant and indicated by asterisks (if not indicated otherwise, test was performed vs. unstimulated control cells for the indicated time point).

**Results**

*S. pneumoniae* R6x induced apoptosis in HUVEC

To study the effect of *S. pneumoniae* on endothelium, HUVEC were infected by unencapsulated *S. pneumoniae* R6x. DNA fragmentation and microscopic morphology were assessed as measure for apoptotic cell death, while non-specific necrosis was estimated by release of lactate dehydrogenase (LDH) into the supernatant. After 16 h of pneumococcal infection, massive DNA fragmentation was observed in HUVEC as shown by nucleosome ELISA (Fig. 1A) and TUNEL stain (confocal laser scanning microscopy, Fig. 1B). The pneumococcal apoptosis signal was higher than the maximal signal obtained by exposure of cells to 1 µM staurosporine within the same time frame (data not shown). We found, that the data obtained by nucleosome ELISA and LDH assay correlated directly and linearly with the number of apoptotic or necrotic cells (data not shown). Moreover, Annexin-V- and propidium iodide-stain (fluorescence microscopy, Fig. 1C) displayed enhanced Annexin-V binding in R6x-infected endothelial cells whereas the propidium iodide uptake was elevated in D39-infected cells. Because most of the R6x infected cells were propidium iodide negative, the data suggest that R6x caused apoptosis whereas D39 provoked necrosis in HUVEC (Fig. 1C). In addition, within the time frame tested, LDH-activity in cell culture supernatants of R6x-infected cells was not significantly increased compared to unstimulated cells (data not shown).

**Endothelial cell apoptosis – effect of encapsulation, pneumolysin, and H₂O₂**

By infecting HUVEC (Fig. 2A, B) and HPMEC (Fig. 2C, D) with encapsulated *S. pneumoniae* strain D39, its unencapsulated derivate R6x and pneumolysin-deficient R6xΔply, we analyzed the impact of pneumococcal virulence factors in more detail. While R6x at 10⁶ cfu/ml induced DNA fragmentation in endothelial cells of both origins, its pneumolysin-deficient mutant R6xΔply induced apoptosis less effectively in HUVEC than in HPMEC. Neither bacteria (R6x, R6xΔply) increased LDH-release within the time frame tested (Fig. 2B, D). In contrast, encapsulated D39 (10⁶ cfu/ml) did not activate endothelial DNA fragmentation within 16 h of exposure, while high levels of LDH-release as a sign of necrotic cell death could be observed. Since *S. pneumoniae* is known to produce H₂O₂ (25, 26) the role of hydrogen peroxide with regard to R6x-induced apoptosis was addressed. Preincubation of HUVEC with NAC significantly reduced DNA fragmentation in R6x-exposed (Fig. 2E), and LDH release in D39-infected cells (Fig. 2F).

**S. pneumoniae** R6x-induced endothelial apoptosis depended on caspase 6 and caspase 9

In many situations, programmed cell death is executed by activation of hierarchical caspase cascades comprising regulatory
Therefore, we analyzed the impact of caspase-activation in R6x pneumococci-induced apoptosis of endothelial cells. The pan-caspase inhibitor zVAD completely inhibited pneumococci-related DNA fragmentation in HUVEC, suggesting an important role of caspases in pneumococci-induced endothelial cell death (Fig. 3A). The used concentration of zVAD did not alter bacterial growth within the time frame tested (data not shown). Next, we assessed activation of caspases 3, 6 and 9 in more detail. In R6x-exposed endothelial cells, increase of activated caspase 6 and cleavage of pro-caspase 9 were observed after 8–12 h, while processing of pro-caspase 3 was first seen after 24 h of pneumococci-infection (Fig. 3B). In a fluorometric caspase activity assay, activation of caspase 6 (Fig. 3D) and 9 (Fig. 3E) was noticed after 16 h of pneumococci-infection. Activation of caspase 3 was observed 24 h after the infection of HUVEC with R6x in the fluorometric caspase activity assay (Fig. 3C, F). In contrast, in staurosporine-stimulated HUVEC strong cleavage of caspase 3 was observed within 16 h (Fig. 3C). Thus, with respect to the observed time-course of apoptosis (Fig. 1), caspase 6 and 9 activity seem to contribute significantly to pneumococci-related endothelial programmed cell death.

**S. pneumoniae** R6x-induced apoptosis reduced expression of anti-apoptotic Bcl-2, and increased cytosolic AIF

The activation of caspases 6 and 9 observed in pneumococci-exposed endothelium (Fig. 3) suggested involvement of mitochondria-dependent factors in pneumococci-related apoptosis (8). Therefore, to expand on this notion, we analyzed protein expression of anti-apoptotic Bcl2 protein, pro-apoptotic Bax protein, as well as AIF, which was shown to be important for caspase-independent apoptosis.

As demonstrated in figure 4, R6x-infection of endothelial cells decreased protein levels of Bcl2 but showed no effect of Bax protein levels within the time frame tested (Fig. 4). Interestingly, we found a strong increase of cytosolic AIF after 8 h of pneumococci infection of HUVEC (Fig. 4).

**Elevation of cAMP blocked S. pneumoniae R6x-induced endothelial apoptosis**

Because cAMP is known to interfere with mitochondria-dependent apoptosis (17), we tested the effect of cAMP-elevation on pneumococci-related endothelial apoptosis (Fig. 5). Endothelial cells degraded cAMP by phosphodiesterase (PDE)-isoenzyme 3 and 4 (27). Simultaneous adenylyl-cyclase-activation (forskolin, 1 μM) and PDE4-inhibition (Rp-73401, 1 μM) resulted in strong accumulation of cAMP-content in HUVEC (data not shown). Elevation of cAMP completely blocked *S. pneumoniae*-induced DNA fragmentation (Fig. 5), and strongly suppressed activation of caspases 6 and 9. No increase in LDH activity in the supernatant could be detected within the time frame studied (data not shown).

**S. pneumoniae** R6x-induced endothelial apoptosis was mediated through p38 MAPK and JNK

MAP kinase signaling pathways were activated in inflammatory reactions and have been shown to play important roles in cell

Figure 3: Caspase-activation in pneumococci-infected endothelial cells. A, HUVEC were pre-incubated with 50 μg/ml pan-caspase inhibitor zVAD for 30 min, and then stimulated with R6x (10^7 and 10^6 cfu/ml) for 16 h. DNA fragmentation was analyzed. B, HUVEC were incubated with R6x (10^5 cfu/ml) for the indicated time periods and active (caspase 6) or pro-forms (caspases 3 and 9) of caspases were detected by Western blot. Representative blots of three independent experiments with similar results were shown. C-F, HUVEC were incubated with R6x (10^7 and 10^6 cfu/ml) for 16 h (C-E) or the indicated time periods (F), respectively, and specific caspase activity (C/F; caspase 3, D; caspase 6, E, caspase 9) was assessed. *, p<0.05 vs. unstimulated control; #, p<0.05 with or without inhibitors; RFU, relative fluorescence units.

Figure 4: Expression of apoptosis-regulating proteins in *S. pneumoniae*-infected endothelium. *S. pneumoniae*-exposure reduced levels of Bcl2 and increased cytosolic AIF but showed no effect on Bax protein levels. HUVEC were incubated with R6x (10^6 cfu/ml) for the indicated time periods and Bcl2, Bax, AIF, and ERK2 (loading control) were detected by Western blot. Representative blots of three different experiments with similar results are shown.
growth and death (28). Therefore we hypothesized that p38 MAPK and JNK may contribute to pneumococci-induced endothelial apoptosis. Phosphorylation of both kinases was up-regulated by R6x-infection in human endothelial cells, as shown by modification-specific antibodies (Fig. 6A). p38 MAP kinase was strongly phosphorylated 30 and 60 min after pneumococci-exposure and phosphorylation disappeared after 2 h. Phosphorylation of JNK persisted up to 2 h. To determine whether MAPK activation is involved in \textit{S. pneumoniae}-induced apoptosis, endothelial cells were pre-incubated with specific inhibitors for p38 MAPK (SB202190) and JNK (SP600125) 1 h before stimulation with R6x-pneumococci (16 h). As shown in figure 6B, SB202190 and SP600125 strongly reduced \textit{S. pneumoniae}-induced DNA fragmentation (Fig. 6B). SB202474, an inactive control compound for SB202190, had no effect. In addition, p38 MAPK (SB202190)- and JNK (SP600125)-inhibitor both greatly reduced pneumococci-dependent caspase 6 (Fig. 6C) and 9 (Fig. 6D)-activity as shown by caspase activity assay. The used concentration of SB202190 and SP600125 did not alter bacterial growth within the time frame tested (data not shown).

\section*{Discussion}

This study demonstrates that unencapsulated \textit{S. pneumoniae} strain R6x induced apoptotic cell death in cultured human endothelial cells. R6x time- and dose-dependently induced programmed cell death, activated regulatory caspase 9 and executor caspase 6, whereas encapsulated D39-pneumococci induced necrosis. Accordingly, a pan-caspase inhibitor completely blocked R6x-induced programmed cell death. Decreased protein levels of Bcl2 and an increase of cytosolic AIF additionally implicated mitochondrial involvement. In accordance, elevation of cAMP significantly reduced caspase-activation and R6x-related DNA fragmentation in human endothelial cells. Generation of H$_2$O$_2$ within the infection contributed to endothelial apoptosis and necrosis. Interestingly, pneumococci activated p38 MAPK and JNK in endothelial cells, and blocking of both kinases reduced pneumococci-related activation of caspases 6 and 9 as well as DNA fragmentation.

Vital endothelial cells are necessary to maintain endothelial barrier function and contributed significantly to the regulation of S. pneumoniae-induced endothelial DNA fragmentation depended on activation of p38 MAPK and JNK. A, HUVEC were incubated with R6x (10$^6$ cfu/ml) for the indicated time periods and phosphorylated p38 MAPK and JNK, or ERK2 (loading control) were detected by Western blot. Representative blots of three independent experiments with similar results are shown. B, HUVEC were pre-incubated with p38 MAPK-inhibitor SB202190 (10 \textmu M), inactive chemical control compound SB202474 (10 \textmu M) or JNK-inhibitor SP600125 (10 \textmu M) and infected for 16 h with \textit{S. pneumoniae} (10$^6$ cfu/ml) and DNA fragmentation was measured. *, p < 0.05 vs. unstimulated control at single time points. C, D, effect of SB202190, SB202474 and SP600125 on \textit{S. pneumoniae}-induced activity of caspase 6 and 9. HUVEC were incubated with R6x (10$^6$ cfu/ml) for 16 h in the absence or presence of SB202190 (10 \textmu M), SB202474 (10 \textmu M), and SP600125 (10 \textmu M), respectively. Specific activity of caspase 6 and 9 was fluorometrically assessed. *, p < 0.05 vs. unstimulated control; #, p < 0.05 with or without inhibitors; RFU, relative fluorescence units.
an inflammatory reaction by e.g. mediator release and regulation of coagulation and fibrinolysis (3). Bacteria-mediated apoptosis of endothelial cells, as shown for pneumococci (this study), E. coli (29), and S. aureus (11, 30)-infection de-stabilized vascular homeostasis (31) and may pave the way for further bacterial invasion and systemic host infection.

Furthermore, loss of endothelial barrier function and subsequent edema formation due to endothelial apoptosis, e.g. in the lung, could result in severe loss of organ function, detrimental for the host. Indeed, Zysk et al. have shown that ingestion of S. pneumoniae in brain microvascular endothelial cells resulted in increased detachment of endothelial cells, an effect that was attributed to cytotoxicity due to pneumolysin (19). Rubin et al. found pneumolysin-induced cytotoxicity of lung endothelium in accordance with our data (32). However, apoptosis or necrosis has not been specifically addressed in these studies. Interestingly, HUVEC showed higher susceptibility against pneumolysin than HPMEC with respect to apoptosis in experiments with pneumolysin-deficient R6x pneumococci. The mechanism underlying this variable response of endothelial cells of different origin is unclear but may be due to varying recognition of pneumolysin by e.g. toll-like receptor 4 (33). Besides endothelial cell apoptosis, Braun et al. found that pneumolysin significantly contributed to neuronal and microglial apoptosis in experimental meningitis (2). Overall, pneumolysin has to be considered as an important virulence factor involved in pneumococci-related apoptosis.

Other important factors implicated in cytotoxicity are pneumococcal encapsulation and H₂O₂-production (25, 31): We found a differential pattern of apoptotic and necrotic cell death by changes in encapsulation: While unencapsulated strain R6x induced DNA fragmentation and annexin V uptake typical for apoptosis, the encapsulated D39 strain caused massive LDH release and high propidium iodide uptake in the absence of DNA-fragmentation and annexin V uptake, indicating predominance of necrosis. The amount of capsular polysaccharide of S. pneumoniae correlated with bacteria virulence in regard to meningitis, sepsis and mortality both in humans and murine models (34–36). Encapsulation of pneumococci had a highly cytotoxic effect on HUVEC (31), identified as necrosis in this study. Similarly, in macrophages (37) and epithelial cells (6), unencapsulated pneumococci are more potent inducers of apoptosis than encapsulated, possibly because encapsulation masks or reduces liberation of apoptosis inducing factors. However, in a different model, Braun et al. found severe neuronal apoptosis in the hippocampus region induced by unencapsulated as well as encapsulated pneumococci (2, 4). In vitro studies addressing molecular mechanisms of pneumococci pathogenesis predominantly used unencapsulated strains due to the highly cytotoxic effects of encapsulated pneumococci (6, 22, 23, 38–41). Therefore, it should be mentioned that further studies are needed to confirm results obtained in vitro with unencapsulated strains in the in vivo situation by using encapsulated bacteria. Since S. pneumoniae-produced H₂O₂ (25) is considered to play a role in pneumococci pathogenesis, the role of hydrogen peroxide with regard to R6x-induced apoptosis was addressed: We demonstrate that NAC significantly reduced DNA fragmentation in R6x-, and LDH-release in D39-infected cells. Other studies found that H₂O₂ produced by pneumococci induced both apoptosis and necrosis in different cell lines (2, 6, 25) and large amounts of H₂O₂ are known to induce apoptosis and necrosis in endothelial cells (42). However, Braun et al. have shown that a strain of S. pneumoniae that produced less than 5% H₂O₂ in comparison to the wild type was fully active in apoptosis induction in neuronal cells (2). Therefore, the observed effect of NAC on DNA fragmentation and LDH release induced by R6x and D39 could be due to detoxification of pneumococci-produced H₂O₂ or the reduction of endothelial production of reactive oxygen species during the infection process (42).

Investigating mechanisms of S. pneumoniae-related programmed cell death in more detail, we found that apoptosis-induction was accompanied by activation of caspases 6 and 9. Consistent with our observations, van Mehring et al. found increased expression of caspases, including caspase 6, in a mouse model of pneumococcal meningitis and sepsis (43). Caspase 6 has been described to play an important role in apoptosis of primary neuronal and endothelial cells (44, 45), e.g. mediating lamin A-cleavage in apoptotic condensation (46). Moreover, caspase 6 was also activated in epithelial cells infected with Pseudomonas aeruginosa (47) and Helicobacter pylori (48). Interestingly in pneumococci-infected endothelial cells pro-caspase 3-cleavage as well as significant caspase 3-activity was only seen at late time points (24 h), after massive apoptosis has already taken place (12–16 h). In contrast, staurosporine-induced caspase 3 activation was noticed after 12 h of infection. However, it is not clear whether a crosstalk between executing caspases led to caspase 6-dependent activation of caspase 3 (49) or whether secondary effects occurred. This suggests that caspase 3 is not necessarily of importance in driving pneumococci-related apoptosis in the experimental model used.

To evaluate the physiological impact of caspase activation in pneumococci-induced endothelial apoptosis, we made use of the pan-caspase inhibitor zVAD. zVAD pre-incubation of endothelial cells strongly reduced pneumococci-induced apoptosis. Caspase inhibition also blocked apoptosis in pneumococci-infected human lung epithelial cells (6) as well as hippocampal apoptosis in experimental pneumococcal meningitis (50). In contrast, pneumococci-infected apoptotic neuronal cells did not show caspase activity, and cell death could not be blocked by caspase-inhibitors (4).

Caspase 9 activation implicates mitochondrial involvement and could be triggered by changes of Bcl2 family proteins, which mediate release of cytochrome C from intermembrane space. Subsequently, cytosolic cytochrome C activates Apaf-1, which oligomerizes caspase 9, leading to activation of downstream caspases such as caspase 3 and 6 (8). Pneumococci-exposure of endothelial cells reduced expression of anti-apoptotic Bcl2 wherever as the level of Bax seemed to be constant within the analyzed time frame, presumably changing steady state towards apoptosis (8). In a previous study we have shown that Bcl2-overexpressing alveolar epithelial cells were largely resistant to S. pneumoniae-induced programmed cell death, suggesting an important role for Bcl2 proteins in pneumococci-dependent apoptosis (6). Similarly, mycobacteria persisting in human macrophages up-regulate Bcl2 to prolong survival of their host cells (51).
Recent reports suggest the existence of caspase-independent apoptotic pathways in the execution of pneumococci-induced cell death (4). Therefore, we determined the release of AIF in endothelial cytosol, which is known to induce caspase-independent apoptosis (4). Pneumococci-exposed endothelial cells demonstrated increased cytosolic AIF levels. In addition, the observed programmed cell death was completely abolished by the pan-caspase inhibitor zVAD. This is strongly indicative of a link between AIF and caspases. This notion is supported by data suggesting that activated caspases and caspase-activated protein t-Bid can trigger release of AIF from mitochondria (52–54). Moreover, in HeLa and Jurkat cell lines treated with stauroporine or actinomycin D, mitochondrial release of AIF is suppressed by caspase inhibitors (55). Therefore, either caspase-independent or caspase-dependent mechanisms of AIF-release from mitochondria may occur. In this respect, it is of interest to note that cAMP-elevating agents (27) blocked S. pneumoniae-related caspase-6 and 9 activation and apoptosis. Whether mitochondria-anchored PKA-dependent phosphorylation and activation of Bad or alternative cAMP-dependent pathways contribute to the rescue of endothelial cells from pneumococci-induced cell death remains to be determined (56).

In this study we show that pneumococci induced strong phosphorylation of p38 MAPK and JNK in human endothelial cells. Inhibition of both pathways significantly reduced S. pneumoniae-related caspase activity, as well as apoptosis of human endothelial cells. p38 MAPK phosphorylation has also been observed in S. pneumoniae-infected lung epithelium (23) and macrophages (57). A phosphorylation of JNK in pneumococci-infection has not been reported until now. In accordance with our observations, Mycobacterium tuberculosis induced p38 MAPK-dependent apoptosis in polymorphonuclear neutrophils (10), and Staphylococcus aureus led to JNK-dependent apoptosis in endothelial cells (11). In contrast, it has been reported that activation of p38 MAPK and JNK prevented endothelial cells death after exposure to Bartonella quintana (58). A possible anti-apoptotic mechanism of p38 MAPK-inhibition could be up-regulation of anti-apoptotic Bcl2 as reported in the hearts of transgenic mice (59). On the other hand, inhibition of p38 MAPK decreased Helicobacter pylori-induced apoptosis without affecting Bcl2 level (60). Taken together, our experimental results suggest, that p38 MAPK and JNK play an important role in pneumococci-induced apoptosis.

In summary, unencapsulated S. pneumoniae induced apoptosis of human endothelial cells, whereas exposure of encapsulated S. pneumoniae led to necrotic cell death. Pneumococci-induced endothelial cell apoptosis by reducing Bcl2 protein, inducing AIF release and subsequent caspase 6- and 9-activation. Precipitation of endothelial cells with NAC reduced pneumococci-induced apoptosis and necrosis. Moreover, activation of p38 MAPK and JNK contributed to pneumococci-related endothelial programmed cell death, and elevation of intracellular cAMP led to strong reduction of S. pneumoniae-induced caspase activation and apoptosis. Therefore, pneumococci-related apoptosis of endothelial cells may have an important role in pneumococci pathogenesis.

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