Apoptosis induced by pneumolysin in human endothelial cells involves mitogen-activated protein kinase phosphorylation

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Abstract. Pneumolysin (Ply) is an essential virulence factor of *S. pneumoniae*, which can induce apoptosis in a variety of host cells to facilitate infection of pathogenic bacteria by as yet unclear mechanisms. To confirm the apoptosis-inducing properties of pneumolysin in endothelial cells, human umbilical vein endothelial cells (HUVECs) were exposed to pneumolysin. The proliferation of HUVECs was inhibited by pneumolysin in a dose- and time-dependent manner. Flow cytometry analysis and ultrastructural changes of the cells indicated the apoptotic response. Exposure to pneumolysin significantly increased the number of apoptotic cells and the activities of caspases-3 and -8. This change was associated with activation of p38 mitogen-activated protein kinase (MAPK) and suppression of extracellular signaling regulation kinase (ERK)1/2. Pre-exposure to the p38 MAPK inhibitor SB-203850 prevented human endothelial cells from apoptosis induced by pneumolysin. In conclusion, these findings demonstrate that pneumolysin induces apoptosis in endothelial cells and the involvement of p38 MAPK activation and ERK1/2 deactivation.

Introduction

S. pneumoniae is one of the most common opportunistic pathogens, which is commonly colonized on the nasopharynx asymptomatically and translocation to lungs, brain and blood leads to pneumonia, otitis media, pneumococcal meningitis and sepsis (1).

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Pneumolysin (Ply) is an essential virulence factor in *S. pneumoniae* (2). Ply has four domains and is composed of 471 amino acid residues, with a molecular weight of 53 kDa (3,4). It belongs to the family of thiol-activated, cholesterol-binding toxins which exert direct cytotoxic effects on host cells by forming ring-shaped transmembrane pores.

Vascular endothelial cells play an important role against bacteria and their virulence factor entry into the blood. It's well known that apoptosis regulation is modulated by multiple phosphorylations of several different protein kinases, including the major subtypes of the mitogen-activated protein kinase (MAPK) family: extracellular signaling regulation kinase (ERK), ERK5, c-Jun N-terminal kinase (JNK) and p38 MAPK (5). It has been shown that ERK is associated with cell survival, whereas p38 MAPK and JNK are involved in apoptosis. However, the involvement of the aforementioned pathway in pneumolysin-induced apoptosis in vascular endothelial cells remains unclear.

Caspases are essential components of the apoptotic response (6). They belong to a group of enzymes known as cysteine proteases and possess the ability to cleave after an aspartate (AsP) residue in their substrate (7). N'Guessan *et al* (8) has confirmed that Ply-induced apoptosis in macrophages is dependent on caspases during pneumococcal infections. In contrast, Ply-induced apoptosis was not involved in the activation of the caspase pathway in dendritic cells (9). In this regard, it is still necessary to clarify whether caspases contribute to pneumolysin-induced apoptosis in vascular endothelial cells.

Exposure of neutrophils to viable pneumococci caused apoptosis and necrosis of the cells, which has been shown to be associated with pneumolysin. The role of pneumolysin in apoptosis is also supported by its ability to induce apoptosis of neuronal cells, dendritic cells, macrophages and cochlear cells (8-11). By comparing *S. pneumoniae* and its isogenic Ply deletion mutant, pneumolysin has been found to be implicated in HUVEC and type-II alveolar epithelial cell apoptosis (8,12).

Therefore, the primary objective of the present investigation was to clarify whether human endothelial cells undergo apoptosis following purified Ply protein exposure and, if so, establish the involvement of the MAPK family and caspase pathways.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection. Cells were cultured in DMEM medium containing 10% (v/v) fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 g/ml) at 37˚C in a humidified atmosphere of 5% CO_2 and the growth medium was changed every other day until cells reached 80% confluence. The deletion of Alanine at the 146 amino acid site of Ply (ΔA146 Ply or Ply mutant) was produced as previously described (13). This mutant has nearly lost its pore-forming activity. Ply was produced as described elsewhere (14).

MTT assay. HUVEC cells were grown to the mid-logarithmic phase and diluted to obtain a 2.5×10^4/ml solution. Cells were then exposed to Ply of various concentrations (0.1, 0.5, 1, 5, 10 μg/ml) for the indicated times (0, 24, 48, 72 and 96 h). Briefly, 200-μl aliquots of cell suspensons were plated on a 96-well plate, and then Ply at the concentrations of 0.1, 0.5, 1, 5, 10 μg/ml was added into the wells and incubated for 24, 48, 72, 96 h, followed by the addition of 20 μl of MTT solution (5 mg/ml). The plate was incubated at 37˚C for 4 h, and the supernatant was removed. The culture medium served as the negative control. The absorbance of the wells, including the blanks, was measured at the wavelength of 492 nm after the addition 150 μl of DMSO to all wells. The experiment was repeated five times. The IC_{50} values were calculated and the cell proliferation inhibitory rate was determined by the equitation: $RI(\%) = [1-A(492)_{treated}/A(492)_{control}] \times 100\%$.

Morphological examination. HUVEC cells were grown to the mid-logarithmic phase and diluted to gain a 2×10^4/ml solution. A volume of 5 ml was inoculated to new culture bottles and cultured for 12 h before any treatment or assay. Ply of 0.5, 1, 2.5 μg/ml was added into the bottles and incubated for 24 h at 37˚C. PBS-treated dishes served as the control. Thereafter, changes in morphology were observed with light microscopy.

Transmission electron microscopy (TEM). The procedure for TEM analysis was performed as previously described (10). Briefly, HUVEC cells were exposed to 1 μg/ml of Ply for 12 h. Thereafter, cells were rinsed twice with PBS, fixed overnight and post-fixed similarly with the procedure for scanning electron microscopy. The cells were placed and allowed to dehydrate through a graded ethanol series and subsequently infiltrated with liquid Araldite resin. Sections were double-stained with uranium acetate and lead citrate. Suitable areas were selected before TEM.

Detection of apoptosis with flow cytometry in vitro. To analyze the apoptosis with propidium iodide (PI) and Annexin V staining, HUVEC cells were exposed to either Ply (0.5, 1, 2.5 μg/ml) or PBS (control). After incubation for 12 h, the cells were harvested according to the manufacturer's instructions, followed by staining with Annexin V-FITC and PI for flow cytoflometry.

Immunocytochemistry. HUVEC cells were incubated with 0.5 μg/ml Ply and PBS for 24 h at 37˚C, pelleted and washed with PBS, fixed (with 4% paraformaldehyde), and permeabilized for 15 min with 3% H_2O_2. These cells were processed for immunocytochemistry with the corresponding antibodies. The p38 MAPK and the ERK1/2 antibodies were diluted 1:50 and 1:250, respectively, in 1% bovine serum albumin, and then cells were stained according to the manufacturer's instructions. Suitable areas were selected for observation of positevely-stained brown particles in the cytoplasm and/or the cell membrane under 10 high-power fields (magnification, x400). A computer image analysis system was used to calculate the mean density (MD) value of the positive cells, and to calculate the ratio, MD (pro)/MD (con), reflecting the level of protein expression.

Fluorometric analysis of caspase activities. HUVEC cells were treated either with Ply at a final concentration of 0.5 μg/ml or with PBS. The absorbance at the wavelength of 405 nm (A405) was measured according to the manufacturer is instructions. The activities of caspase-3, -8 and -9 were calculated as: Activity = $A_{treated}/A_{control}$.

Western blotting. HUVECs were incubated with 0.5 μg/ml Ply or PBS for 12 h. Cells were pelleted and washed with PBS for three times. These cells were lysed on ice in lysis buffer containing 10 mg/ml protease inhibitor (Roche, Mannheim, Germany) by gently shaking twice. The supernatant was obtained by 12,000 r/min of centrifugation at 4˚C for 10 min. The protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene membrane. Blots were blocked and incubated with rat anti-human p38 MAPK or ERK1/2 primary antibodies(Cell Signaling Technology) or an anti-β-actin antibody (Santa Cruz Biotechnology, Inc.) overnight at 4˚C. Blots were rinsed, incubated with a horseradish peroxidase-conjugated secondary antibody, and developed using the ECL kit (Millipore, Billerica, MA, USA).

Statistical analyses. All data are presented as the mean ± SD and were analyzed with the SPSS 17.0 software. A value of P<0.05 was considered statistically significant.

Results

Penumolysin inhibits the proliferation of HUVECs. The viability of cells in the confluent monolayer of HUVECs was markedly suppressed by the different concentrations of Ply treatments for the indicated test periods when compared to the negative control with no pneumolysin treatment, as shown by the MTT data (Fig. 1A) (P<0.05). The growth of HUVEC cells was inhibited by pneumolysin in a dose- and time-dependent manner. The effect of pneumolysin (0.5, 1, 2.5 μg/ml) on the HUVEC morphology was observed under light microscopy (Fig. 1B). Significant morphological changes of HUVECs exposed to pneumolysin were observed. In the control dishes, monolayer cells were spindle-shaped, adherent, and showed typical 'cobblestone-like' morphological characteristics. When Ply was added, the micrographs show HUVECs had deviated from their typical appearance demonstrating that the number of viable cells was negatively-dependent on Ply concentrations.
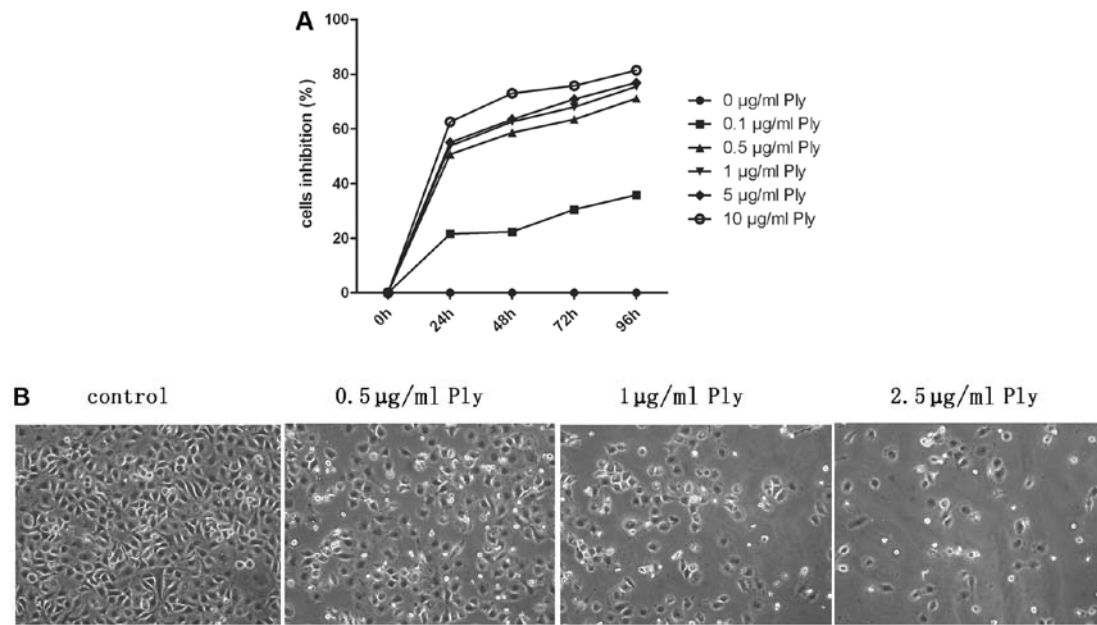
Figure 1. Effects of pneumolysin on HUVEC cell survival, proliferation and cell morphology (x100). (A) HUVECs were exposed to various concentrations of Ply (0.1, 0.5, 1, 5, 10 µg/ml) for 0, 24, 48, 72 and 96 h. The growth of HUVECs was markedly suppressed in the Ply treated groups compared to the Ply mutant group or the control cells (P<0.05). The growth of HUVECs was inhibited by Ply in a dose- and time-dependent manner. (B) HUVECs were exposed to Ply for 12 h with indicated doses. In control dishes, cells showed the typical 'cobblestone-like' morphological characteristic. When exposed to pneumolysin, cells became winkled and the cell number decreased.
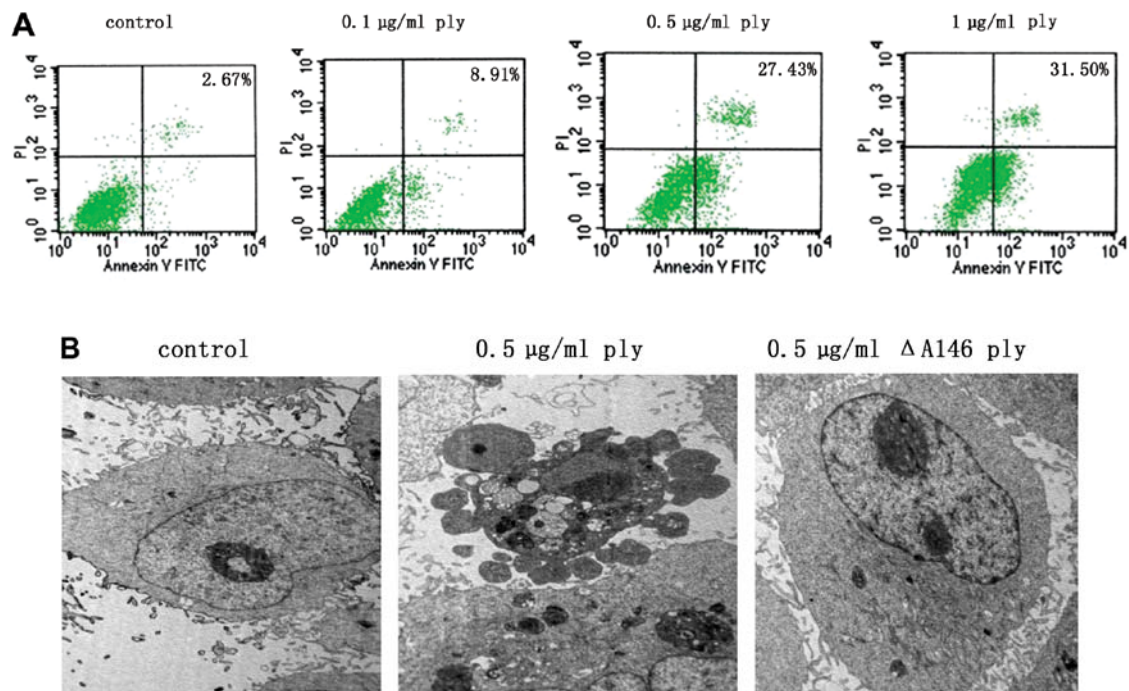

Figure 2. Apoptosis induced by pneumolysin. (A) Induction of apoptosis by pneumolysin as measured by flow cytometry. The HUVECs were treated with 0.1, 0.5 and 1 µg/ml of pneumolysin or PBS for 12 h. The apoptosis rates for 0.1 µg/ml pneumolysin treated group was 8.91%, and when the concentration increased to 0.5 and 1 µg/ml, the apoptosis rates were elevated to 27.43 and 31.50%, respectively. A significant difference was observed compared to the control cells (P<0.05). (B) Apoptosis of HUVECs observed by transmission electron microscopy (x8,000). HUVECs were exposed to 0.5 µg/ml pneumolysin, 0.5 µg/ml ΔA146 Ply or PBS for 12 h.

Apoptosis of HUVECs exposed to pneumolysin. Considering Ply could inhibit the proliferation of HUVECs, we investigated its role in apoptosis induction of HUVECs. The HUVECs were treated with 0.1, 0.5 and 1 µg/ml of Ply or PBS for 12 h

(Fig. 2A). The apoptosis rate was detected with flow cytometry after Annexin V/PI staining. The apoptosis rate for the 0.1 µg/ml Ply-treated group was 8.91%, and the apoptosis rates were increased to 27.43 and 31.50% at the 0.5 and 1 µg/ml
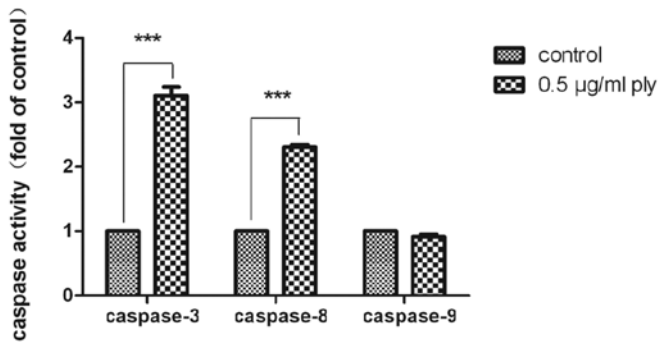
Figure 3. Activities of caspases-3, -8 and -9. Following 12 h culture, HUVEC cells were treated either with Ply at a final concentration of 0.5 μg/ml or PBS as indicated. The activities of caspases-3, -8 and -9 were calculated as: Activity = $A_{treated}/A_{control}$. Values are compared between stimulated and non-stimulated cells. ***P<0.0001. Each bar represents the mean ± SD of separate experiments.

Ply concentrations, respectively. A significant difference was observed compared to the control cells (P<0.05). TEM was carried out at the concentration of 0.5 μg/ml Ply. After 12 h exposure, typical apoptosis characteristics such as bubbles in the cytoplasm, chromatin condensation and apoptosis bodies were observed under TEM (Fig. 2B).

Pneumolysin induces the activities of caspases-8, -9, -3. Caspases play an important role in apoptosis. We therefore, investigated whether they are involved in the apoptosis of

HUVECs induced by Ply using caspase activity assays. Incubation of HUVECs with 0.5 μg/ml Ply led to the activation of caspases-8 and -3, but not caspase-9. Caspases were not activated in the controls (Fig. 3).

Activation of p38 MAPK and deactivation of ERK1/2. Next, we addressed whether the effects on HUVECs by Ply correlated with the activation and phosphorylation of p38 MAPK and ERK1/2. Using immunochemical assays, p38 MAPK and ERK1/2 were examined in HUVEC cells (Fig. 4A). Ply exposure led to a significant increase (P<0.05) in the phosphorylation of p38 MAPK compared with the control group. After Ply exposure, ERK1/2 in HUVECs was reduced compared to the control cells (Fig. 4B, C and D).

Pneumolysin-induced apoptosis of HUVECs could be blocked by p38 MAPK inhibitor. HUVEC cells were pretreated with 20 μM SB-203580 (obtained from Cell Singaling Technology) (a p38 MAPK inhibitor) for 1 h. The presence of the p38 MAPK inhibitor resulted in a significant decrease of the apoptosis rate in the Ply treatment group (Fig. 5) (P<0.05).

Discussion

Pneumolysin is an important exotoxin released by *S. pneumoniae*. Previous studies had reported that, exposure of neutrophils, macrophages, and epithelial cells to pneumolysin caused apoptosis and necrosis of the cells (8,9,13). The present study demonstrated the apoptosis of HUVEC following
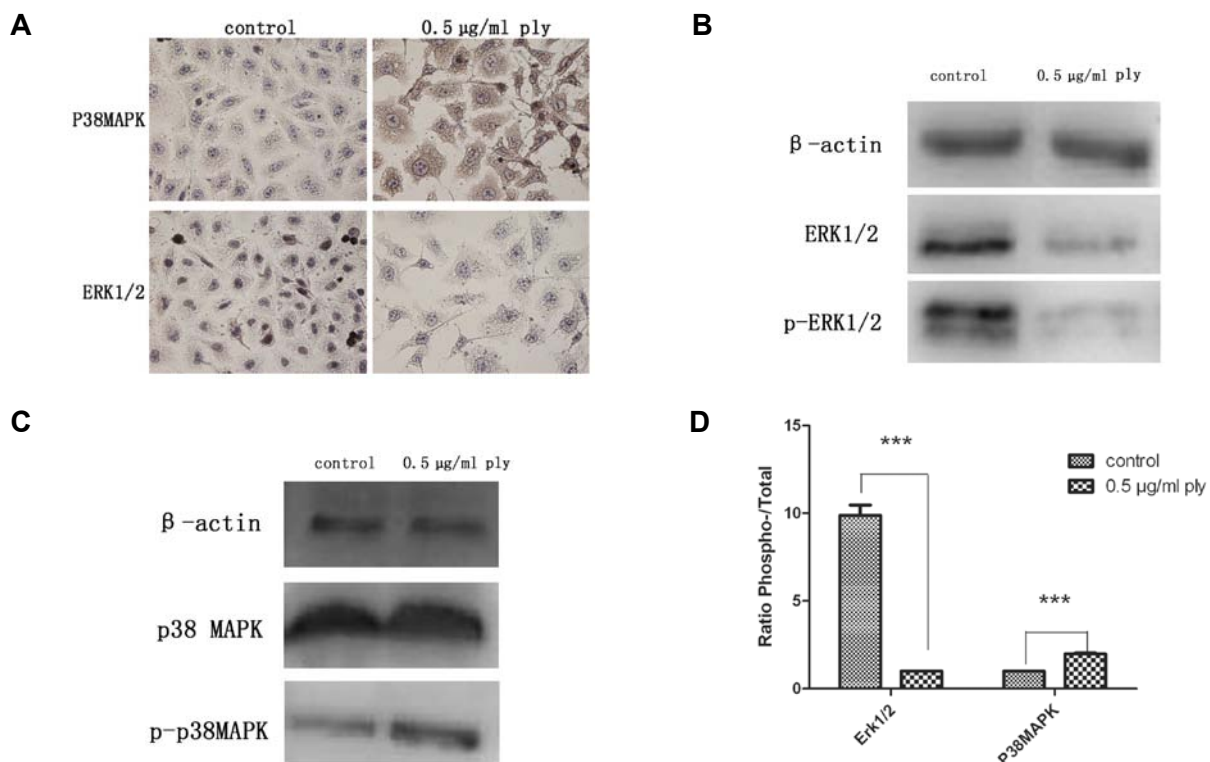

Figure 4. Ply induced the activation of p38 and reduced that of ERK 1/2. (A) HUVEC cells were incubated with 0.5 μg/ml Ply and PBS (control) for 24 h at 37°C (magnification, x400). Using immunocytochemical assays, p38 MAPK and ERK1/2 expression was examined in HUVEC cells. (C and D) Pneumolysin exposure led to a significant increase (P<0.05) in phospho-p38 MAPK compared to the control group. (B and D) After Ply exposure, phospho-ERK1/2 in HUVECs was reduced compared to the control cells. Values are compared between stimulated and nonstimulated cells. ***P<0.0001. Each bar represents the mean ± SD of separate experiments.
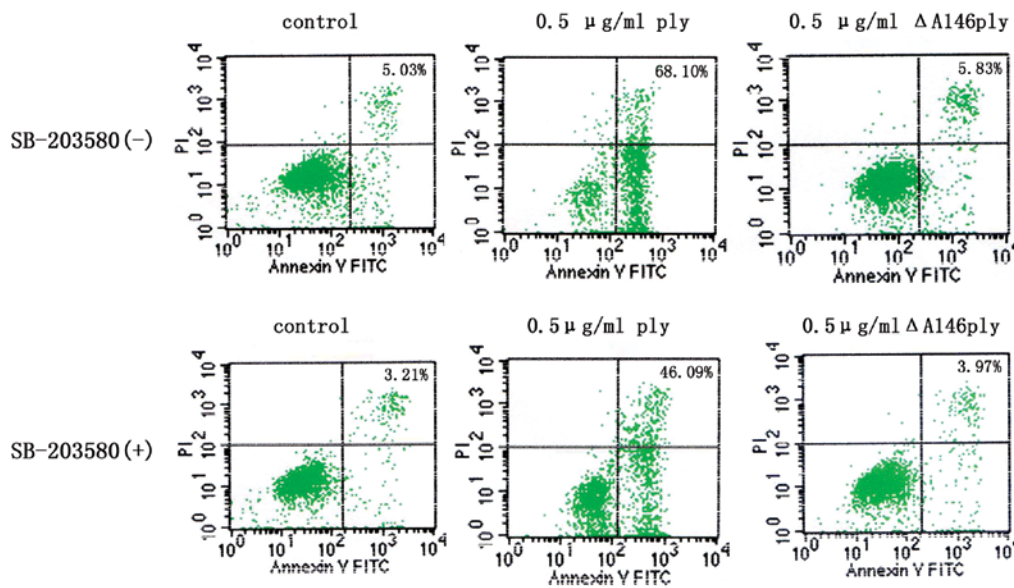
Figure 5. Effect of the p38 MAPK inhibitor SB-203850 on Ply-induced apoptosis. HUVECs were pretreated with 20 μM SB-203580 (a p38 MAPK inhibitor) for 1 h before treatment with 0.5 μg/ml of pneumolysin for 12 h. Upper panel, no. SB-203580. The apoptosis rate was significant higher in Ply-exposed cells compared to control. Lower panel, pretreated with SB203580 followed by Ply exposure. The addition of SB-203580 significantly decreased the apoptosis rate induced by Ply (P<0.05).

pneumolysin exposure, and thus expanded the knowledge on pneumolysin induced apoptosis.

Basically, the endothelium forms a physical barrier that separates blood from tissue, and maintains as well as regulates barrier function against various kinds of stimuli such as inflammation (14). Moreover, they involve the earliest events in inflammation, such as the adhesion of immune cells (15). It is well known that ICAM-1, VCAM-1 and E-selectin are inducible by cytokines such as TNF-α during infections, and contribute to an increase in adhesion and chemokine release in endothelial cells (16). Thus, loss of endothelial barrier functions due to endothelial apoptosis, e.g. in the vasculature, could result in bacterial invasion and cause systemic bacterial infection.

In the present study, the effect of pneumolysin on HUVECs was clearly demonstrated by light microscopy and TEM observations. From the images, one can clearly see that HUVECs underwent morphological changes, from the typical spindle-shaped, to ones that had wrinkled and round shape; the cell number decreased (Fig. 1B). Besides, apoptosis was observed under TEM, and further confirmed by Annexin V/PI staining. Hahne *et al* (17) reported that pneumolysin interaction with brain microvascular endothelial cells led to increased detachment of cells in accordance with our results. Therefore, pneumolysin-mediated apoptosis of endothelial cells, as shown in this study may pave the way for further invasion and eventually systemic infection.

Cell apoptosis is a critical process controlling the host balance under normal or stress conditions. The modulation of apoptosis was related to a series of complex caspase reactions. There are two typical caspase-associated pathways leading to apoptosis: one involves the activation of caspase-8, while the other is caspase-9 dependent and involves the mitochondria. Both pathways will eventually activate caspase-3, initiate the entire caspase cascades, and hence induce apoptosis. It

is controversial whether pneumolysin-induced apoptosis is dependent on caspases. Therefore, we determined the activity of caspases-3, -8 and -9, which constitute the two typical apoptosis-associated pathways. HUVECs demonstrated increased activity of caspases-3 and -8, whereas caspase-9, implicating mitochondrial involvement, was not activated in this study. This suggests that pneumolysin-induced apoptosis in HUVECs occurs through caspase-8 (extrinsic pathway) but not caspase-9 (intrinsic pathway).

MAPK kinase signaling pathways were activated in response to the inflammation response. p38 and ERK1/2 are two major downstream MAPK cascades (18,19). Therefore, we hypothesized that p38 and ERK1/2 may contribute to Ply-induced endothelial cell apoptosis. In the present study, pneumolysin promoted not only the activity but also the phosphorylation of p38 MAPK. In contrast, not only the activity but also the phosphorylation of ERK1/2 was inactivated in HUVECs. It is not clear whether a crosstalk between the two signaling pathways led to the apoptosis induced by pneumolysin. However, we demonstrated that activated p38 is required for pneumolysin-induced apoptosis, as shown by the ability of the p38 inhibitor to prevent cells from undergoing apoptosis.

In conclusion, this study provides evidence in support of the involvement of p38 MAPK in Ply-induced HUVEC apoptosis and the apoptosis is dependent on the activation of caspases-3 and -8.

Acknowledgements

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