Original Article

Role of α-toxin-induced apoptosis of umbilical vein endothelial cells in vertical infection of Staphylococcus aureus L-form

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Abstract: Objective To investigate α-toxin-induced apoptosis of umbilical vein endothelial cells and explore its role in vertical infection of Staphylococcus aureus L-form. Methods HUV-EC-C cells exposed to different concentrations (0, 10, 30, 90, and 270 ng/ml) of α-toxin for different time lengths (0, 2, 4, 6, and 8 h) were examined for apoptosis using flow cytometry with Annexin V-FITC staining. The levels of vascular necrosis factor-α (TNF-α) and the activities of caspase-3 and caspase-8 in the cell culture were detected by ELISA and colorimetric method, respectively. α-Toxin-induced cell apoptosis was also analyzed in HUV-EC-C cells treated with a neutralizing antibody of TNF-α or with the inhibitory peptides of caspase-3 (zDEVD-FMK) and caspase-8 (zIETD-fmk). Results α-Toxin induced apoptosis of HUV-EC-C cells in a dose- and time-dependent manner and caused significantly enhanced expression of TNF-α and the activation of both caspase-3 and caspase-8. Inhibition of TNF-α with its neutralizing antibody and the inhibitory peptides of caspase-3 or caspase-8 all significantly decreased α-toxin-induced cell apoptosis, and the caspase-3 inhibitor completely blocked α-toxin-induced cell apoptosis. Conclusion α-Toxin-induced apoptosis is partially mediated by the extrinsic cell death pathway of TNF-α and caspase-8 and plays an important role in the vertical infection of S. aureus L-form to affect fetal growth and development.

Key words: umbilical vein endothelial cells; α-toxin; vertical infection; Staphylococcus aureus; L-form

INTRODUCTION

Staphylococcus aureus (S. aureus) is a common human pathogen carried by 25%-35% of the population and can potentially cause severe infections in pregnant women and infants [1]. The recent years have seen an increasing prevalence of methicillin-resistant S. aureus (MRSA) worldwide in both the health care [2-5] and community settings [6,7]. MRSA has been reported as an emerging problem among pregnant women due to its colonization at such sites as the genital tract, breast, buttocks, vulva and groin [8] to facilitate its vertical transmission from the mother to the infant. Vertical transmission of MRSA also occurs in postpartum women [9]. Although several studies have shown that MRSA colonization among pregnant women in the third trimester and in postpartum women exposes the neonates or infants to an increased risk of vertically transmitted infection, little is known about the mechanism by which MRSA colonization during pregnancy affects the fetuses in the uterus.

Normally S. aureus is not able to pass through the placental barrier, but a cell-wall-deficient variant strain of S. aureus, known as S. aureus L-form, can emerge spontaneously in vivo under certain pathological conditions [10,11]. Clinical and laboratory data have indicated that L-form bacteria frequently contribute to in vivo bacterial survival and persistence [10,11], and are associated with atypical, chronic or latent infections [12]. Compared with its parental strain, S. aureus L-form has a smaller size and morphological plasticity [13,14] to facilitate its passing through the placental barrier to cause fetal infection as virus does.

Our previous studies [13,14] suggested that S. aureus L-form was capable of passing through the placental barrier to affect fetal growth and development, but how it affects the fetus remains unclear. Similar to S. aureus, S. aureus L-form also produces α-toxin, a soluble poreforming toxin which induces apoptosis in epithelial cells [15], peripheral blood mononuclear cells [16], endothelial cells [17] and Jurkat T cells [18]. In this study, we aimed to investigate the effect of α-toxin on umbilical vein endothelial cells lining the internal surface of the umbilical vein. We chose HUV-EC-C cells, an endothelial cell line originally derived from human umbilical vein, to examine α-toxin-induced apoptosis of umbilical vein endothelial cells and explore its role in the vertical transmission of S. aureus L-form.

MATERIALS AND METHODS

Reagents

S. aureus α-toxin was purchased from Sigma-
Aldrich (St Louis, MO). Polyclonal anti-human TNF-α antibody was purchased from PeproTech (Rocky Hill, NJ). Caspase-3 inhibitory peptide zDEVD-FMK, caspase-8 inhibitory peptide zIETD-fmk, and caspase-3 and-caspase-8 colorimetric assay kits were supplied by BioVision (Mountain View, CA). Human tumor necrosis factor-α (TNF-α) ELISA kit was obtained from Biolegend (San Diego, CA). Annexin V-FITC staining kit was the product of BD Biosciences (Heidelberg, Germany).

Cell line and culture

HUV-EC-C (ATCC CRL-1730) cells were maintained in Ham’s F12K medium with 2 mmol/L L-glutamine (containing 1.5 mg/ml sodium bicarbonate, 0.1 mg/ml heparin, 0.03-0.05 mg/ml endothelial cell growth supplement, 10% heat-inactivated fetal bovine serum, and 50 μg/ml streptomycin and penicillin) at 37 °C in a 5% CO₂ humidified atmosphere. Assays were performed in serum-free Ham’s F12K medium. In some experiments, Ham’s F12K medium was supplemented with various inhibitors, antibody, and/or different concentrations of S. aureus α-toxin as specified.

Cell treatments with α-toxin, antibody and various inhibitors

The cultured cells were counted and plated at a density of 1×10⁵/ml in 6-well tissue culture plates. Upon cell adhesion to the plates, the culture medium was changed to serum-free Ham’s F12K medium, and different concentrations of S. aureus α-toxin were added for treatment for 8 h. After centrifugation at 1500 r/min for 10 min, the culture supernatants and cell pellets were collected for measuring TNF-α by ELISA kit according to the manufacturer’s protocols and determining cell apoptosis and caspase activities, respectively. The cells were also exposed to treatments with anti-human TNF-α, zDEVD-FMK or zIETD-fmk at the final concentrations of 20 μg/L, 2 mmol/L, and 2 mmol/L, respectively, and the cell apoptosis was analyzed with flow cytometry. The cells without induction were used as the control in all the assays.

Detection of apoptosis by flow cytometry

To observe apoptosis-related changes on the plasma membrane, phosphatidylserine was detected on the cell surface using annexin V, and the cell membrane permeability was assessed with propidium iodide using Annexin V staining kit according to the manufacturer’s instructions. Briefly, 1×10⁶/ml cells were exposed to serum-free medium alone or to various inhibitors and antibody in the presence or absence of different concentrations of S. aureus α-toxin. The cells were then washed and re-suspended in 0.1 ml staining solution (2% Annexin V-FITC and 2% propidium iodide-PE in HEPES buffer), and incubated for 15 min in darkness at room temperature. Flow cytometry was performed on a FACS Calibur (Becton Dickinson, Heidelberg, Germany) using CellQuest software. For each determination, a minimum of 50 000 cells was analyzed. Early apoptotic cells were positively stained with annexin V but not with propidium iodide, and late apoptotic or necrotic cells were positively stained with both.

Colorimetric determination of caspase-3 and -8 activities

The activities of caspas in the cells were determined using Caspase Colorimetric Assay Kits according to the manufacturer’s protocols. Briefly, the cell lysates were incubated for 2 h at 37 °C with 5 μL of 4 mmol/L N-acetyl-Asp-Val-Asp-aminomethyl-coumarin (DEVD)-p-nitroanilide (pNA) for caspase-3 and N-acetyl-Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8 in 50 μl reaction buffer containing 50 mmol/L HEPES (pH 7.4), 100 mmol/L NaCl, 10% sucrose, 0.1% CHAPS, and 10 mmol/L DTT. Based on the chromophore pNA after cleavage from the labeled substrate DEVD-pNA or IETD-pNA, pNA light emission was quantified using a spectrophotometer at 405 nm. Comparison of the absorbance of pNA from a test sample with the control sample allowed determination of the fold increase in caspase activity.

Statistical analysis

The results are expressed as Mean ± SD. Turkey’s-b in One-way ANOVA was used to assess the significance of differences within experiments. A P value less than 0.05 was considered to indicate a statistical significance.

RESULTS

Apoptosis of HUV-EC-C cells exposed to α-toxin

Exposure to different concentrations of α-toxin for 8 h caused dose-dependent apoptosis in HUV-EC-C cells (Fig.1), and the cell apoptosis reached the peak level after exposure to 30 ng/ml α-toxin. Hence, 30 ng/ml α-toxin was chosen to investigate the time course of apoptosis in the HUV-EC-C cells. HUV-EC-C cells incubated with 30 ng/ml α-toxin showed time-dependent increases in cell apoptosis rates (P<0.05, Fig.1).

α-Toxin-induced cell apoptosis was partially mediated by TNF-α

HUV-EC-C cells incubated with different concentrations of α-toxin for 8 h showed significantly higher levels of TNF-α production than the control cells (Fig.2A). Addition of TNF-α antibody in the cell culture prior to α-toxin exposure significantly attenuated α-toxin-induced cell apoptosis (Fig.2), but the cell
Fig. 1  *S. aureus* α-toxin induced apoptosis in HUV-EC-C cells in a dose- and time-dependent manner. **A**: Flow cytometric analysis of HUV-EC-C cells exposed to different concentrations of α-toxin for 8 h; **B**: Apoptotic rates of HUV-EC-C cells exposed to different concentrations of α-toxin for 8 h; **C**: Changes of HUV-EC-C cell apoptotic rates after exposure to 30 ng/ml α-toxin for different time lengths. The data represent the average values from 5 independent experiments. Error bars represent the standard deviations. *P<0.01 vs vehicle (0 ng/ml α-toxin).

Fig. 2 α-Toxin-induced cell death is partially mediated by TNF-α. **A**: TNF-α production in the cells culture detected by ELISA after an 8-h exposure to different concentrations of α-toxin; **B**: Flow cytometric analysis of HUV-EC-C cell apoptosis induced by α-toxin in the absence or presence of TNF-α antibody; **C**: Apoptotic rates of HUV-EC-C cells incubated with 30 ng/ml α-toxin in the absence or presence of TNF-α antibody for 8 h. The data represent the average values from 5 independent experiments. Error bars represented standard deviations. *P<0.01 vs vehicle; "#"P<0.01 vs α-toxin alone.
apoptotic rates were still higher than the control level.

**α-Toxin activated caspase-3 and caspase-8 in HUV-EC-C cells**

Exposure to α-toxin for 8 h dose-dependently increased the activities of both caspase-3 (Fig.3) and caspase-8 (Fig.4) in HUV-EC-C cells. Exposure to 30 ng/ml α-toxin resulted in the maximum caspase-3 and -8 activation. Addition of caspase-3 inhibitory peptide zDEVD-FMK and caspase-8 inhibitory peptide zIETD-fmk in the cell culture prior to α-toxin significantly decreased α-toxin-induced cell death, but the cell apoptotic rates were still higher than the control level (Fig.3 and Fig.4).

**DISCUSSION**

α-Toxin is one of the important pathogenic substances produced by both *S. aureus* and its L-form. We demonstrated in this study that α-toxin induced apoptosis of HUV-EC-C cells in a dose- and time-dependent manner. Hypothetically, the damage of the endothelial cells lining the maternal umbilical vein induced by α-toxin can cause injuries of the umbilical venous vessels to result in disturbances of oxygen and nutrient supplies to the fetus, which leads consequently to fetal growth and development abnormalities.
As a human commensal pathogen, *S. aureus* is a major cause of community and nosocomial infection. In pregnant and postpartum women, MRSA resides commonly in the breasts, buttocks, vulva, and groin.[10] MRSA colonization in the maternal genital tract exposes the neonate to an increased risk for early-onset invasive neonatal infection, but little is known whether the vertical transmission of *S. aureus* affects the fetus other than the neonate. Our previous studies[11-14] showed that *S. aureus* L-form, by vertical transmission, could cause fetal growth and development retardation in mice. In this study, we further explored the role of α-toxin in vertical infection of *S. aureus* L-form. The results showed that α-toxin could induce apoptosis of the vascular endothelial cells in *vitro*, which may be one of mechanisms by which vertically transmitted *S. aureus* L-form affects fetal growth and development.

Cell apoptosis is mediated essentially by a family of intracellular cysteine proteases known as caspases.[20-22] Activation of caspases is achieved via two principal signaling pathways, namely the extrinsic and intrinsic death pathways.[22-23] Upon formation of the death-inducing signaling complex by the extrinsic death pathway or the apoptosome by the intrinsic death pathway, pro-caspase-8 or pro-caspase-9, respectively, are autoproteolytically processed, resulting in the activation of the downstream caspases. Our finding that α-toxin stimulated TNF-α expression in HUV-EC-C cells was consistent with a recent report that TNF-α expression was up-regulated in both epithelial cells[15] and peripheral blood mononuclear cells exposed to *S. aureus* α-toxin. In addition, the removal of TNF-α with a neutralizing antibody significantly decreased α-toxin-induced cell death, but failed to completely block cell apoptosis, suggesting that TNF-α partially contributes to α-toxin-induced apoptosis through the extrinsic death pathway.

Given that α-toxin initiated cell apoptosis by TNF-α binding with the host’s TNF-α receptors, the downstream caspase pathways were also investigated. Studies have shown that *S. aureus* and α-toxin can activate the effector caspase-3 and the two major initiator caspases (caspase-8 and caspase-9) as well.[19,24]. Consistent with these studies, our results also showed that α-toxin could induce the activation of both caspase-3 and caspase-8. Meanwhile, the inhibition of either caspase-3 or caspase-8 with their inhibitors significantly decreased α-toxin-induced cell death, but the caspase-3 inhibitor could completely block the α-toxin-induced cell death. These findings show that caspase-8 of the extrinsic death pathway partially mediates the apoptosis of HUV-EC-C cells induced by *S. aureus* α-toxin.

In conclusion, α-toxin can induce HUV-EC-C cell apoptosis *in vitro* in a dose- and time-dependent manner, and plays an important role in the vertical infection of *S. aureus* L-form to affect fetal growth and development. α-Toxin-induced expression of TNF-α partially mediates HUV-EC-C cell death by activating both caspase-3 and caspase-8.

Acknowledgments

We wish to thank Dr. LI Baiqing in the Department of Immunology, Benghu Medical College for assistance in FACS analysis.

REFERENCES

α-毒素诱导的脐静脉内皮细胞凋亡在金葡菌L型垂直感染中的作用

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摘要:目的 确定α-毒素诱导脐静脉内皮细胞(HUV-EC-C)的凋亡在金葡菌L型垂直感染中的作用。方法 在培养的HUV-EC-C细胞中加入不同浓度(0, 10, 30, 90, 270 ng/ml)的金葡菌α-毒素,处理不同时间(0, 2, 4, 6, 8 h)后经Annexin V-PI染色,流式细胞仪检测HUV-EC-C细胞的凋亡率。通过ELISA检测α肿瘤坏死因子(TNF-α)、caspase-3与caspase-8的表达量,并观察加入TNF-α中和抗体、caspases-3抑制剂zDEVD-FMK、caspase-8抑制剂zIETD-fmk对α-毒素诱导HUV-EC-C细胞凋亡的影响。结果 α-毒素能够诱导HUV-EC-C细胞凋亡并表现于时间、剂量依赖性,明显增加HUV-EC-C细胞中TNF-α、caspase-3与caspase-8的表达;在加入TNF-α中和抗体、zDEVD-FMK、zIETD-fmk后可部分阻断α-毒素诱导的HUV-EC-C细胞凋亡。结论 α-毒素通过TNF-α及caspase-8介导的外源性死亡途径诱导HUV-EC-C细胞凋亡,表明α-毒素诱导内皮细胞凋亡是金葡菌L型垂直感染影响胎儿发育的主要机制之一。

关键词:脐静脉内皮细胞;α-毒素;垂直感染;金葡菌;L型

2013-01-04

基金项目:国家自然科学基金(81070506)

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