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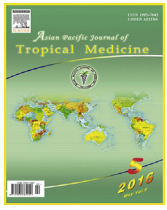

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Study on the role of Cathepsin B and JNK signaling pathway in the development of cerebral aneurysm

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ABSTRACT

Objective: To investigate the correlation between JNK signal and the apoptosis of VSMC as well as the expression of Cathepsin B and to explore the role of JNK signal in the development of cerebral aneurysm.

Methods: Rat models of cerebral aneurysm were established and histopathologic changes of cerebral aneurysm and the apoptosis of VSMC were analyzed. Rat models were respectively subject to subcutaneous injection of Cathepsin B siRNA and JNK inhibitor SP600125. Western blot technique was used to detect the expression of proteins like Cathepsin B, Caspase-3, and p-JNK. Spearman's rho was used to examine the correlation between p-JNK and Cathepsin B, as well as the expression of relevant proteins.

Results: The success rate of modeling rats with cerebral aneurysm was 88.75%. After the respective injection of Cathepsin B siRNA, SP600125 and their combination, the cell densities of VSMC of rats with cerebral aneurysm all increased significantly ($P < 0.05$ or $P < 0.01$), but the apoptosis rate of VSMC decreased significantly ($P < 0.01$). Compared with normal rats, the expression of Cathepsin B, Caspase-3 and p-JNK in Cerebral aneurysm models increased significantly. Effectively intervening Cathepsin B genes with Cathepsin B siRNA could significantly inhibit the expression of Cathepsin B and Caspase-3, but hardly influence the expression of p-JNK. JNK inhibitor SP600125 had no influence on the expression of Cathepsin B and Caspase-3, but effectively inhibited the expression of p-JNK. In cerebral aneurysm tissues, positive correlation was observed between the expression of p-JNK and Cathepsin B, the correlation coefficient was $r = 0.640$.

Conclusion: After the attack of cerebral aneurysm, proteins like Cathepsin B, Caspase-3 and p-JNK are all involved in the apoptosis of VSMCs. This process may be realized by Cathepsin B which activates the apoptosis mechanism of Caspase-3 and mediate the apoptosis of VSMC through the JNK signaling pathway. Therefore, silencing Cathepsin B gene or inhibiting the conduction through JNK signaling pathway can mitigate the apoptosis of vascular smooth muscle cells in cerebral aneurysm.

1. Introduction

Cerebral aneurysm is a common cerebrovascular disorder and its rupture usually leads to high rate of disability and/or death [1]. Regarding the pathogenesis of cerebral aneurysm, current studies mainly focus on genetic factors, hemodynamic factors, and acquired degenerative changes in arterial walls [2–4]. However, it's also significant to explore an effective way to intervene the growth of cerebral aneurysm by studying its pathogenesis from the perspective of molecular biology. Normally, the proliferation and apoptosis of VSMC help to maintain the balance and stability in blood vessels. However, the occurrence of cerebral aneurysm breaks such balance, causing the apoptosis of a large number of VSMCs, as a result of which weakened walls of blood vessels in the brain fail to sustain the impact of blood flow [5]. Previous study showed that excessive apoptosis of VSMC is an important mechanism for the formation of cerebral aneurysm [6].

Cathepsins are enzymes in lysosomes, including Cathepsin B, C, K, L, and S and others which play a vital role in the

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process of apoptosis. Cathepsin B is a lysosomal cysteine protease. A wide range of diseases results in elevated levels of Cathepsin B, which causes pathological processes of numerous diseases like cancer, inflammation, and degenerative diseases. The influence of Cathepsin B on apoptosis has also been widely studied [7]. Caspase-3 is the most critical protease in cell apoptosis and an activator of the execution-phase of cell apoptosis [8]. c-Jun N-terminal kinases (JNKs) belong to the mitogen-activated protein kinase family (MAPK) and JNK signaling pathways play a vital role in a variety of physiological processes like cell differentiation, apoptosis and stress [9]. Phosphorylated JNKs (p-JNK) eventually cause a series of certain biological effects by regulating downstream signaling molecules. SP600125, an inhibitor of JNKs, can effectively inhibit the conduction of JNK signaling pathways [10]. By establishing the mouse model of cerebral aneurysm, this study explored the apoptosis rate of VSMC and changes in the expression of Cathepsin B, Caspase-3 and p-JNK. This study also analyzed the influence on cerebral aneurysm by effectively intervening Cathepsin B or blocking JNK signaling pathways in order to find out the role of Cathepsin B and JNK signals in the pathogenesis of cerebral aneurysm.

2. Material and methods

2.1. Establishment of the rat model of cerebral aneurysm

A total of 80 male Sprague–Dawley rats, 5–7 weeks old, provided by Laboratory Animal Center of Zhengzhou University, were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg). Subsequently, the rats were subjected to the ligation of left common carotid artery and the bipolar coagulation of posterior branches of bilateral renal arteries. One week after the procedures, the rats were fed with 1% saline instead of water until the 12th week. The cerebral vessel wall tissues were separated from the right ACA-OA under the microscope, fixed with 4% paraformaldehyde in 0.1 M PBS for 24–48 h, subject to paraffin embedding, sectioning, and hematoxylin-eosin staining. Finally, histopathologic changes were observed. TUNEL assay was used to detect the cell density and apoptosis rate of VSMC. This experiment has been approved by Laboratory Animal Ethics Committee of Zhengzhou University.

2.2. Grouping and treatment of experimental animals

A total of 50 rat models of cerebral aneurysm were equally divided into 5 groups, namely the scramble siRNA group subject to subcutaneous injection of Scramble siRNA, the Cathepsin B siRNA group subject to subcutaneous injection of Cathepsin B siRNA, the SP600125 group subject to subcutaneous injection of SP600125, the Cathepsin B siRNA + SP600125 group subject to subcutaneous injection of Cathepsin B siRNA + SP600125, and the model control group subject to subcutaneous injection of equivalent normal saline.

2.3. TUNEL assay used to detect the cell density and apoptosis rate of VSMC

Deparaffin cerebral vessel wall tissues of all groups with dimethylbenzene and ethanol till all the paraffin was replaced by water. Use Proteinase K to digest the tissues at room temperature, use 3% H_2O_2 in methanol to block the activity of endogenous horseradish peroxidase, add TdT and digoxin-labeled dUTP for mixture reaction, incubate the tissues, add horseradish peroxidase-labeled anti-digoxin antibody for incubation, use DAB-H_2O_2 solution to stain. Cells with dark brown nuclei were in the process of apoptosis. Finally, restain, dehydrate, clear, and mount the tissues. For negative control groups, replace TdT solution with PBS buffer. Count the number of cells in certain visual fields under 400X microscope, calculate cell density. Given 200 cells in each visual field, calculate the percentage of cells in apoptosis, namely cell apoptosis rate.

2.4. Western blotting

Extract the total protein of tissues of all groups, use 50 μg protein for 15% SDS-PAGE, then transfer proteins to PVDF membrane, use 5% nonfat milk in TBST buffer as a blocking agent, incubate overnight at 4 °C respectively with Cathepsin B rat anti-human polyclonal antibody (1:200), Caspase-3 rabbit anti-human polyclonal antibody (1:300), rat anti-human p-JNK monoclonal antibody (1:400), and β-actin rat anti-human monoclonal antibody (1:200), then incubate with HRP-labeled goat anti-rat second antibody (1:500) at 37 °C for 1 h, rinse the membrane with TBST, then use electrochemical luminescence (ECL) assay to observe the results. All antibodies were bought from Santa Cruz Biotechnology Inc. The same method was used to detect the expression of p-JNK and Cathepsin B in the other 21 cerebral aneurysm rat models.

2.5. Statistical analysis

Software SPSSl7.0 was used for One-way ANOVA of relevant data. Spearman's rho was used to analyze the correlations between the expression of p-JNK and Cathepsin B. All the data were represented with Mean ± SD. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Establishment of the rat model of cerebral aneurysm

Under light microscope, 30 of 71 rats have aneurysm-like lesions, all of which were at the intersection of anterior cerebral artery-olfactory artery (ACA-OA) on the contralateral side of the circle of Willis with the success rate of modeling being 88.75%. HE staining showed that normal arteries had complete endothelial cells and smooth muscle fibers and tunica adventitia were neat and compact, while in cerebral aneurysm arteries collagenous fibers of cerebral aneurysm thickened, smooth muscle layer atrophied, tunica adventitia was loose, and there was thrombosis at the lumen of the aneurysm.

3.2. Detecting the cell density and apoptosis rate of VSMC

In Situ cell death detection with TUNEL assay showed that compared with the model control group, VSMC cell densities of the Cathepsin B siRNA group, the SP600125 group, and the Cathepsin B siRNA + SP600125 group all increased

significantly ($P < 0.05$ or $P < 0.01$), but decreased significantly compared with that of normal rats ($P < 0.01$). Compared with normal rats, the VSMC apoptosis rate of the cerebral aneurysm model control group increased significantly ($P < 0.01$). Compared with the cerebral aneurysm model control group, VSMC apoptosis rates of the Cathepsin B siRNA group, the SP600125 group, and the Cathepsin B siRNA + SP600125 group decreased significantly ($P < 0.01$), but were all higher than that of normal rats ($P < 0.01$) (Table 1).

3.3. Detecting the expression of relevant proteins

There was weak or no expression of Cathepsin B in the tissues of cerebral vessel wall of normal rats, but significantly increased expression of Cathepsin B was observed in cerebral aneurysm models. Effectively intervening Cathepsin B genes with Cathepsin B siRNA could obviously inhibit the expression of relevant proteins. However, JNK inhibitor SP600125 had no influence on the expression of Cathepsin B. Caspase-3 was expressed in normal tissues, but cerebral aneurysm caused significantly higher expression level of Caspase-3. The expression of Caspase-3 in cerebral aneurysm models was inhibited by the injection of Cathepsin B siRNA, but not influenced by the injection of JNK inhibitor SP600125. p-JNK was expressed in normal tissues, but more significantly expressed in cerebral aneurysm tissues. Cathepsin B siRNA had no significant influence on the expression of Cathepsin B; however, JKN inhibitor SP600125 effectively inhibited the expression of p-JNK (Table 2).

3.4. Correlation between the expression of p-JNK and Cathepsin B

The expression of p-JNK and Cathepsin B in cerebral aneurysm models subject to no treatment was respectively examined. Spearman's rho was used to analyze the correlation between the expressions of them. Positive correlation was observed between the expression of p-JNK and Cathepsin B, the correlation coefficient was $r = 0.640$, $P = 0.002$. According to the fitting degree of the trend line, the coefficient of determination (R^2) = 0.589.

Table 1
Cell density and apoptosis rate of VSMC.

Group	Cell density (SMCs/HPF)	Apoptosis rate (%)
Normal control	225.84 ± 11.02	3.23 ± 0.06
Model control	37.96 ± 2.01$^{*\#}$	31.05 ± 1.04**
Scramble siRNA	40.21 ± 2.54**	28.66 ± 0.87**
Cathepsin B siRNA	68.76 ± 3.01$^{**\#}$	17.09 ± 2.41$^{**\#}$
SP600125	72.05 ± 5.46$^{**\#}$	15.87 ± 1.77$^{**\#}$
Cathepsin B siRNA + SP600125	123.36 ± 10.12$^{**\#}$	9.52 ± 1.48$^{**\#}$

$^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* normal control group.
$^{\#}P < 0.05$ *vs.* model control group.

Table 2
Relative expression of Cathepsin B, Caspase-3 and p-JNK.

Group	Cathepsin B	Caspase-3	p-JNK
Normal control	0.085 ± 0.02	0.354 ± 0.11	0.202 ± 0.06
Model control	0.539 ± 0.21**	0.764 ± 0.18**	0.498 ± 0.13*
Scramble siRNA	0.519 ± 0.14**	0.805 ± 0.25**	0.487 ± 0.08*
Cathepsin B siRNA	0.032 ± 0.01$^{\#\#}$	0.148 ± 0.09$^{*\#\#}$	0.492 ± 0.22*
SP600125	0.535 ± 0.23**	0.771 ± 0.32**	0.056 ± 0.01$^{**\#}$
Cathepsin B siRNA + SP600125	0.041 ± 0.05$^{\#\#}$	0.057 ± 0.03$^{**\#}$	0.037 ± 0.05$^{**\#}$

$^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* normal control group.
$^{\#}P < 0.05$, $^{\#\#}P < 0.05$ *vs.* model control group.

4. Discussions

The incidence of 2%–3% of cerebral aneurysm leads to an annual incidence of 0.6‰–2‰ of subarachnoid hemorrhage (SAH) [11,12]. Endothelial dysfunction is the initial symptom of cerebral aneurysm, followed by the phenotypic modulation of VSMCs, extracellular matrix remodeling, the apoptosis of VSMC and the degeneration, expansion and rupture of the vessel wall [13]. However, the specific pathogenesis remains unclear. This study investigated the role of Cathepsin B, Caspase-3 and JNK signaling pathway, which are closely related to cell apoptosis, in the attack and development of cerebral aneurysm from the perspective of molecular biology.

Normally, Cathepsin B lies in lysosomes and is involved in physiological processes, like the growth of the body. However, when the cellular injury leads to increased permeability of lysosome membrane or even the rupture of lysosome membrane, Cathepsin B will enter the cytoplasm or surrounding tissues, be activated and mediate inflammatory necrosis and apoptosis of cells [14,15]. Experimental results showed that the expression of Cathepsin B and Caspase-3 was significantly higher in cerebral aneurysm rat models than in normal rats, and the injected siRNA inhibited not only the expression of Cathepsin B but also that of Caspase-3. This suggests that during the attack of cerebral aneurysm, Cathepsin B may cause the apoptosis of cells by activating Caspase-3. However, no final conclusion has yet been reached on the specific mechanism of Cathepsin B activating Caspase-3. Some research has shown that Cathepsin B can directly catalyze Caspase precursor to activate it thus causing cell apoptosis [16]. Some studies conclude that Cathepsin B indirectly activates Caspase, firstly by triggering mitochondrion through Bid to release cytochrome c, then activating Caspase family to cause apoptosis [17,18]. However, both approaches finally work on Caspase-3, the effector of apoptosis, which cause apoptosis together with the apoptosis substrate. Tsubokawa *et al.* [19] found that in the rat model of ischemia-reperfusion injury, the expression of Cathepsin B in the ischemic cortex increased and caused an increased expression of Caspase-3 via bcl-2 family members, thus influencing the apoptosis of neurocytes. Moreover, some study found that the expression of Cathepsin B during the apoptosis of neurocytes in the rat model of ischemia-reperfusion injury increased and it influenced the apoptosis of neurons through signaling pathway or other ways [20].

According to results of this study, in cerebral aneurysm tissues, the expression of p-JNK and Cathepsin B both increased significantly and positive correlation was observed. However, Cathepsin B siRNA had no influence on the expression of p-JNK, neither did JNK inhibitor SP600125 on the expression of Cathepsin B. The specific correlation between Cathepsin B and JNK signaling pathway has not being clear yet. There is a research proving that JNK signaling pathway is an important pro-apoptotic pathway [21]. However, Cathepsin B is involved in cell apoptosis in a quite complicated way. It may mediate the apoptosis either through one protein or certain signaling pathway [22,23]. Therefore, we conclude that in cerebral

aneurysm Cathepsin B may mediate the apoptosis of vascular smooth muscle cells through JNK signaling pathway.

Conflict of interest statement

We declare that we have no conflict of interest.

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