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Expression change of interleukin-8 gene in rabbit basilar artery after subarachnoid hemorrhage

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Abstract: Objective To study the expression change of interleukin-8 (IL-8) gene in the basilar artery of rabbit and the effect of IL-8 on the development of cerebral vasospasm induced by experimental subarachnoid hemorrhage (SAH). **Methods** Thirty five healthy Japanese White Rabbits were randomly divided into saline-control group and experimental group. The experimental group was subdivided into four groups, representing day 1, 4, 7 and 14 after the first blood injection of SAH. The delayed cerebral vasospasm (DCVS) model was established by double injection of autologous blood into the cisterna magna. The expression change of cytokine IL-8 mRNA in the basilar artery was analyzed by RT-PCR. **Results** The expression of IL-8 gene increased on day 4-7 after the first blood injection of SAH compared with control ($P < 0.001$), and decreased to normal on day 14. The expression of IL-8 gene in the SAH groups were positively correlated with the degree of basilar artery stenosis ($r = 0.642$, $P < 0.01$). **Conclusion** The expression level of IL-8 gene in basilar arteries was intimately associated with the degree of cerebral vasospasm, suggesting that IL-8 may play an important role in the DCVS after SAH as an immunological inflammatory factor.

Keywords: intracranial vasospasm; interleukin-8; RT-PCR; subarachnoid hemorrhage

1 Introduction

Delayed cerebral vasospasm (DCVS) after subarachnoid hemorrhage (SAH) is a major cause of mortality and morbidity in SAH patients. Its pathogenesis, however, is still ambiguous, and no completely effective clinical therapy is available currently. The important effect of immunological inflammatory on vessel walls in the pathogenesis of vasospasm has been noticed in recent years[1,2], but the researches about the mRNA expression change of the inflammatory factors in the cerebral vascular were scarcely reported.

In this study, we established a rabbit DCVS model after SAH by double injection of autologous blood into the cisterna magna. By using RT-PCR we investigated the dynamic change of interleukin-8 (IL-8) mRNA in the basilar artery, as well as the relationship of IL-8 and DCVS, and then discussed the possible molecular mechanism of DCVS.

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2 Materials and methods

2.1 Establishment of the DCVS model and animal grouping Thirty-five healthy Japanese White Rabbits (2000-2500 g, Wenzhou Medical College Laboratory Animal Center) were divided into normal saline group and SAH group. The SAH was subdivided into four groups: SAH-1, SAH-4, SAH-7 and SAH-14, representing day 1, day 4, day 7 and day 14 after the first blood injection of SAH, respectively. There were seven rabbits in each group.

Anaesthetized with intramuscular injection of ketamine hydrochloride (25 mg/kg) and chlorpromazine (12.5 mg/kg), the rabbit was fixed on the operating table. A midline occipital incision was made to expose the atlanto-occipital region clearly. An 18-gauge trocar was inserted into the cisterna magna at an angle of 30° with the body and advanced for about 1 cm. Withdraw the stylet after a sense of breakthrough to confirm the presence of clear cerebral spinal fluid. Then 0.5 mL/kg cerebral spinal fluid was aspirated. Next, 1 mL/kg non-heparinized blood drawn from the middle artery of rabbit ear was injected into the cisterna magna slowly. To facilitate the injected blood to cumulate at the cranial base, the rabbit was tilted at 30° for 15 min with the head down. This day was recorded as day 0 after SAH.

Forty-eight hours later, we repeated a second blood injection with the same method as above.

For the control group, the same amount of normal saline was injected into the rabbit cisterna magna. After the digital subtraction angiography (DSA), the animals were reanaesthesized and perfused transcardilly. Subsequently, the rabbit basilar artery was taken out and divided into two parts, one was saved at -70 °C for RT-PCR, and the other was fixed in 10% formalin.

2.2 DSA examination Cranial DSA examination for the control group and the experimental groups (SAH-1, SAH-4, SAH-7 and SAH-14) was processed to observe the degree of basilar artery vasospasm. Basilar arteries were assessed with Holland Philips 3D Digital Subtracting System with Uitravist 370 as the contrast agent. Make an incision at the right inguinal regioned and isolate the femoral artery. A 4F radiography catheter was inserted into the femoral artery and advanced to the left subclavian artery. Guided by the roadmap, the micro-catheter with micro-guidewire was advanced into the intervertebral foramen segment of the left vertebral artery (V2 segment), and then the micro-guidewire was withdrawn. Heparin saline, followed by the contrast agent (1 mL), was injected into the micro-catheter. The basilar artery angiography was performed afterwards. The degree of basilar artery vasospasm was calculated by the diameter method: Percentage of stenosis = (1-minimal diameter/ mean diameter of the distal and the proximal vascular of the stenosis) ×100%.

2.3 Materials harvest The rabbits were perfused transcardilly with 10% formalin for 30 min immediately after DSA. The whole brains were taken out and immerged in 10% formalin for 24 h. Then the rabbit basilar arteries were dehydrated and embedded by paraffin. Finally, the slices in the coronal plane were stained by hematoxylin-eosin.

2.4 Detection of IL-8 mRNA expression

2.4.1 Extraction of total RNA from rabbit basilar artery Total RNA was extracted strictly according to the manufacturer's instructions of TaKaRa RNAiso Reagent (TaKaRa Co., Japan). The absorption values of diluted RNA at A260 and A280 were measured by UV spectrophotometer, with which we calculated purity and concentration of the original RNA.

2.4.2 Quantification of IL-8 mRNA expression by RT-PCR RT-PCR was carried on a PCR cycler (Eppendoff, Germany) by using the BcaBESTTM RNA PCR Kit (Ver. 1.1, TaKaRa Co., Japan). The whole process was in accordance with the manufactory's instructions strictly. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as an inner reference. Primers were determined according to the reported literatures[3,4]. The primer sequences for IL-8 were: forward: 5'-CAT GGA TCT GTC GTA GGG CT-3', reverse: 5'-CTG ACC AAC AGA CCA GGG TT-3' (with a desired product of 390-bp); and for GAPDH were: forward: 5'-TCA CCA TCT TCC AGG AGC GA-3', reverse: 5'-CAC AAT GCC GAA GTG GTC GT-3' (with a desired product of 293-bp).

Conditions for PCR amplification was: initial denaturing at 94 °C for 5 min, followed by denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and extending at 72 °C for 30 s; for 35 cycles. Final extension was at 72 °C for 5 min.

Photos of the PCR product electrophoresis in 2% agarose (0.5×TBE, 5 V/cm) was recorded by a gel imaging analysis system (Parmacia Biotec Co., Ltd). Data of the PCR products were analyzed by the BandScan software (Glyko Inc., USA). The ratio of IL-8 to GAPDH served as a semi-quantitative index of IL-8 mRNA.

2.5 Statistical analysis Data were presented as mean ± SD. The statistical analysis software was SPSS13.0 for Windows. Differences between groups were analyzed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

3 Results

3.1 The morphological characters of the basilar artery vasospasm In SAH groups, narrowing of the vascular lumens, thickening of the smooth muscle layers, and infiltrating of numerous inflammatory cells in the arterial walls were observed under the microscope. While in the control group, the vascular endothelial cells covered the fibrous ring of the internal elastic lamina smoothly. There was no corrugation of the internal elastic fibers. The smooth muscle layer was thin. Very a few inflammatory cells scattered into the vascular walls (Fig. 1).

3.2 Changes of the degree of basilar artery vasospasm Basilar artery and vertebral artery of the control group can be seen clearly by DSA. While in the experimental groups, the rabbit basilar arteries blurred and the arterial walls ruffled on day one after the first blood injection. Vasospasm peaked on day 4 and day 7, associated with bean shape and vascular occlusion on DSA. Until day 14, the basilar artery image had not recovered completely (Tab. 1, Fig. 2).

3.3 Changes of IL-8 mRNA expression in rabbit basilar artery In the control group, small amount of IL-8 mRNA could be detected in the basilar artery. IL-8 mRNA increased

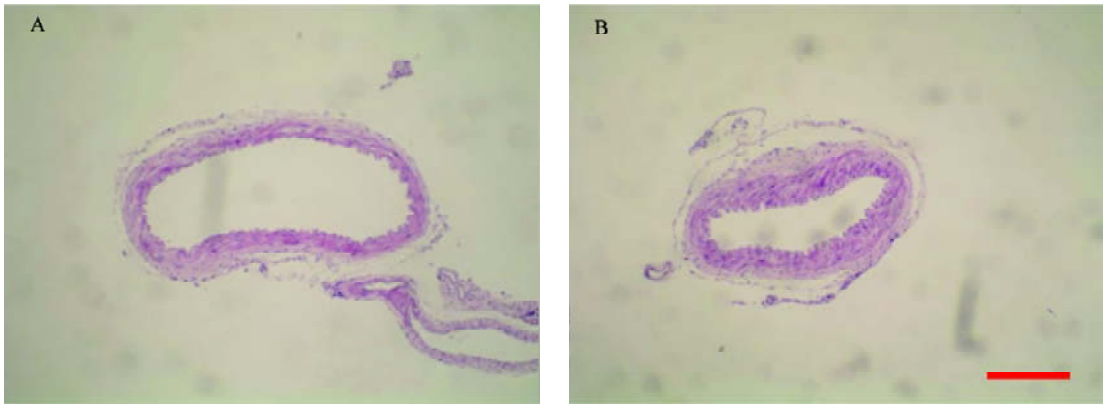

Fig. 1 Microphotograph of the rabbit basilar artery in the normal control (A) and the SAH-7 group (B). Differing from the control group, in SAH groups, narrowing of the vascular lumens, thickening of the smooth muscle layers, and infiltrating of numerous inflammatory cells in the arterial walls could be observed under the microscope. Scale bar, 0.5 mm.

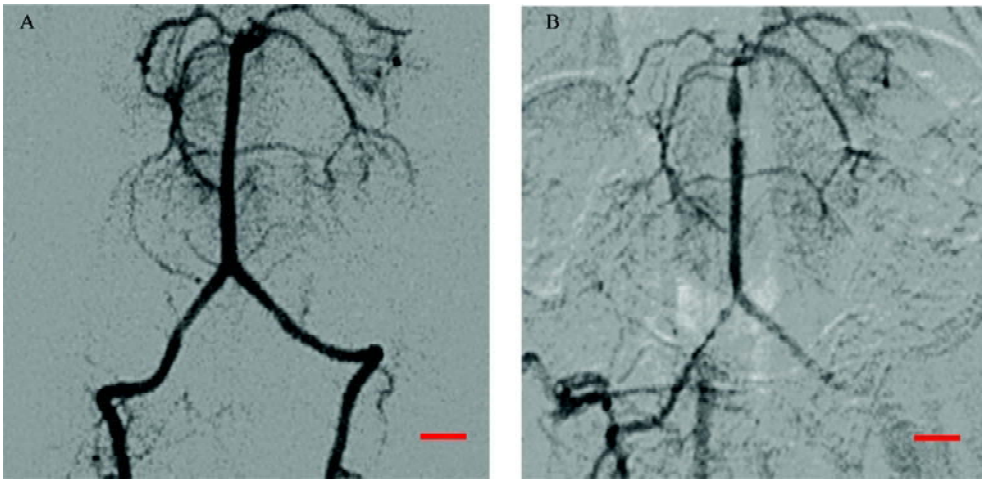

Fig. 2 Digital subtraction angiography (DSA) of the vertebral artery and the basilar artery. The vertebral artery and basilar artery of the rabbit without blood injection appeared clearly in DSA (A); while the basilar artery contracted distinctly and the vertebral artery blurred on day 7 after the blood injection (B). Scale bar, 3.6 mm.

Tab. 1 Dynamic change of basilar artery stenosis in rabbit delayed cerebral vasospasm model

Group	Normal saline	SAH-1	SAH-4	SAH-7	SAH-14
Stenosis (%)*	4.9±2.9	5.4±3.4	33.3±12.1^{A}	40.0±6.6^{A}	11.2±5.3

Note: * Percentage of stenosis = (1 – minimal diameter / mean diameter of the distal and the proximal vascular of stenosis) ×100%. $n = 7$ for each group. ^{A} $P < 0.001$ *vs* Normal saline.

Tab. 2 Dynamic change of IL-8 mRNA in the basilar artery of rabbit delayed cerebral vasospasm model

Group	Normal saline	SAH-1	SAH-4	SAH-7	SAH-14
mRNA index	0.13±0.07	0.21±0.10	0.76±0.13^{A}	0.82±0.16^{A}	0.33±0.12^{B}

Note: $n = 7$ for each group. ^{A} $P < 0.001$, ^{B} $P < 0.01$ *vs* Normal saline

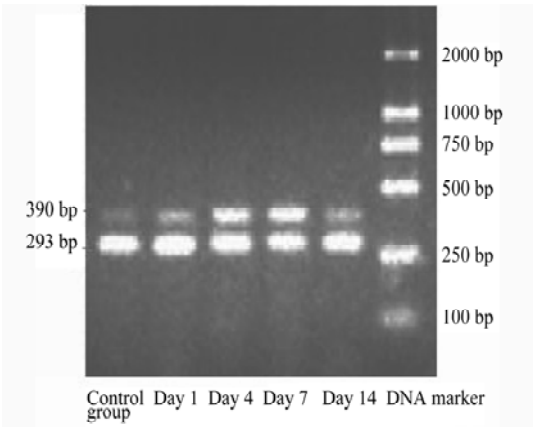

Fig. 3 Expression change of IL-8 gene. IL-8 mRNA expression increased on day 1 after SAH, reached the peak on day 4 and day 7, then decreased to normal on day 14.

on day 4-7 after SAH ($P < 0.05$ *vs* control group), then decreased to normal level on day 14 ($P < 0.01$ *vs* control group) (Tab. 2, Fig. 3).

3.4 The relationship between IL-8 mRNA and basilar artery vasospasm The IL-8 mRNA level in the SAH groups were positively correlated with the degree of basilar artery vasospasm ($r = 0.642$, $P < 0.01$).

4 Discussion

IL-8 is a pleiotropic cytokine with important biological functions in the complicated cytokine network of body system. It recruits neutrophilic granulocytes, accelerates vascular growth, and induces smooth muscle cells to proliferate and migrate[5]. IL-8 is an important immunological inflammatory regulatory factor. Many experiments and clinical studies suggested that the immunological inflammatory responses induced by SAH took part in the development of cerebral vasospasm[1,2]. The responses were also intimately associated with cerebral ischemia[6]. The expression of IL-8 gene increased significantly after SAH, revealing that IL-8 plays an important role in the development of DCVS.

Bowman *et al*.[7] simulated the cerebral vessels after SAH in the rat femoral artery model. The rat femoral artery was immerged in autologous blood. Levels of cytokines were determined by enzyme-linked immunosorbent assay. The results indicated that various inflammatory cytokines increased following SAH, particularly the IL-8, which increased remarkably. Aihara *et al*.[8] measured the expression of various inflammatory factors in the SAH dog by using quantitative PCR. They reported that the expression of IL-8 gene were intimately associated with the degree of cerebral vasospasm and the time phase of inflammatory reaction. In our experiments, the pathological changes of the ischemic vessels in the SAH groups included vessel contraction, prominent vascular smooth muscle layer thickening and inflammatory cell infiltration in the vascular wall. However, there were no significant changes of vessels in the control group. These results indicate that during the development of DCVS, IL-8, as an important chemokine, recruits lots of neutrophilic granulocytes into the vascular walls, after which various vasoconstriction substances are released. Incrassation of vascular smooth muscle and destruction of vascular walls finally resulted in DCVS. The facts that IL-8 mRNA was elevated on day day 4-7 after the SAH and decreased to normal on day 14 showed that the expression of IL-8 was not only changed with time but also positively correlated with the degree of cerebral vasospasm ($r = 0.642$, $P < 0.01$). The dynamic change of IL-8 mRNA was parallel to the degree of cerebral vasospasm. In accordance with the report of Aihara *et al*.[8], our results suggested that IL-8 was tightly associated with the development of DCVS.

It remains unknown how IL-8 exerts its effect in the DCVS. One possible mechanism is that IL-8 may act as a kind of chemokine to recruit various inflammatory cells, especially neutrophilic granulocytes, to infiltrate into the vascular wall. The infiltration led the destruction and pathological hyperplasia of vascular walls, and final narrowing of vascular lumen. On the other hand, IL-8 accelerated the proliferation and incrassation of the vascular smooth muscles, which may also exacerbate the vasospasm. Presently, to some degree Nimodipine could prevent the cerebral vasospasm after SAH, but its effect is not recognized completely. Many researchers begin to use immunological inflammatory suppression method to prevent DCVS, which seems effective[9,10]. Taken together, IL-8 plays a prominent role in the development of DCVS. Suppressing the inflammatory and immunological responses by IL-8 antagonists could be a possible new way to treat and prevent the cerebral vasospasm in SAH patients.

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蛛网膜下腔出血后白细胞介素 -8 基因在兔脑基底动脉中表达的变化

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摘要：目的 探讨实验性蛛网膜下腔出血(SAH)诱发脑血管痉挛时，白细胞介素-8(IL-8)基因在兔脑基底动脉中表达的变化及在诱发脑血管痉挛中的作用。**方法** 35只健康日本大耳白兔随机分为生理盐水组、SAH组。SAH组根据第一次注血时间又分为四组，分别为第一次注血后第1、4、7、14天。以枕大池二次注血法构建迟发性脑血管痉挛模型，采用RT-PCR法观察兔基底动脉中细胞因子IL-8 mRNA表达的变化。**结果** IL-8mRNA在SAH组第一次注血后第4－7天升高，14天趋于正常。SAH组IL-8的表达水平与基底动脉的狭窄程度呈正相关($r = 0.642$，$P < 0.01$)。**结论** IL-8在基底动脉中的表达水平与脑血管痉挛的程度紧密相关，提示IL-8可能作为免疫/炎症因素因素参与了SAH后迟发性脑血管痉挛的发生。

关键词：迟发性脑血管痉挛；白细胞介素 -8；逆转录 - 聚合酶链反应；蛛网膜下腔出血