

# Expression of NF- $\kappa$ B, MCP-1 and MMP-9 in a Cerebral Aneurysm Rabbit Model

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**ABSTRACT: Objective:** We explored the early expression of NF- $\kappa$ B, MCP-1 and -MMP 9 in a rabbit carotid aneurysm model, and investigated the possible mechanism of aneurysm. **Methods:** Twenty four adult New Zealand rabbits were divided into four groups. Normal control (Group A); Rabbits received elastase induction for 1, 2 3 weeks (Group B, C and D respectively); Hematoxylin-Eosin stains were performed for observation. The mRNA and protein expression of NF- $\kappa$ B, MCP-1 and MMP-9 were analyzed using RT-PCR and immunohistochemical methods. **Results:** The expression of NF- $\kappa$ B and MCP-1 reached their peaks after induction for one week, then decreased. Their expression in week 1 and week 2 had no statistical difference. The expression of MMP-9 increased after induction. We observed the highest expression at week 3. As the induction time increased, the number of smooth muscles reduced. Endothelial cells were damaged; the aneurysm wall elastic layer was damaged. **Conclusion:** Activation of NF- $\kappa$ B may be one of the initiating factors contributing to the occurrence and development of cerebral aneurysm. MCP-1 induced macrophage adhesion and infiltration in the artery wall of cerebral aneurysms, and contributed to the occurrence and development of brain aneurysm. Damage to elastic fibers is one of the key factors for aneurysm formation. Increased infiltration of inflammatory cells and the secretion of MMP-9 are the main reasons for elastic fiber damage.

**RÉSUMÉ: Expression de NF- $\kappa$ B, MCP-1 et MMP-9 dans un modèle d'anévrisme cérébral chez le lapin. Objectif :** Nous avons exploré l'expression précoce de NF- $\kappa$ B, MCP-1 et MMP-9 dans un modèle d'anévrisme carotidien chez le lapin et nous avons étudié les mécanismes possibles de la formation des anévrismes. **Méthode :** Vingt-quatre lapins adultes de Nouvelle-Zélande ont été divisés en 4 groupes : le groupe A était le groupe témoin ; les groupes B, C et D ont reçu une induction par l'élastase pendant 1, 2, et 3 semaines respectivement. Des colorations HE ont été effectuées. L'ARNm et l'expression de NF- $\kappa$ B, MCP-1 et MMP-9 a été analysée au moyen de techniques de RT-PCR et d'immunohistochimie. **Résultats :** L'expression de NF- $\kappa$ B et de MCP-1 atteignait son sommet après induction d'une durée d'une semaine puis diminuait. L'expression à la semaine 1 n'était pas différente au point de vue statistique de celle observée à la semaine 2. L'expression de MMP-9 augmentait après l'induction et l'expression la plus élevée était observée à la semaine 3. À mesure que le temps d'induction augmentait, le nombre de cellules musculaires lisses diminuait. Les cellules endothéliales étaient endommagées et la couche élastique de la paroi de l'anévrisme était endommagée. **Conclusion :** L'activation de NF- $\kappa$ B pourrait être un des facteurs déclenchants qui contribuent au développement des anévrismes cérébraux. L'adhésion des macrophages induite par MCP-1 et leur infiltration dans la paroi artérielle des anévrismes cérébraux contribuent à l'apparition et au développement des anévrismes cérébraux. Le dommage aux fibres élastiques est l'un des facteurs clés de la formation des anévrismes. Une augmentation de l'infiltration par des cellules inflammatoires et la sécrétion de MMP-9 sont les principales causes du dommage observé au niveau des fibres élastiques.

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Brain aneurysms are also referred to as cerebral aneurysms or intracranial aneurysms (IA). Rupture of an IA is a major cause of subarachnoid hemorrhage<sup>1</sup>. Cerebral aneurysms are most commonly found at arterial bifurcations and the outer bends of curved vessels. Aneurysm pathophysiology is complex and remains poorly understood. Chronic inflammation and pathological remodeling of arterial wall extracellular matrix are reported to be important mechanisms for the occurrence and development of the aneurysm<sup>2</sup>. During the aneurysm development, various constituents of the inflammatory response appear to be involved, including adhesion molecules, cytokines, reactive oxygen species, leukocytes, matrix metalloproteinases (MMPs), and vascular smooth muscle cells<sup>3</sup>. Nuclear factor kappa B is a key molecule that is involved in the vascular inflammation of IA<sup>4,5</sup>. Upregulated monocyte chemoattractant protein-1 (MCP-1) has been reported in IA patients. Zhang *et al*

indicated that the level of monocyte chemoattractant protein-1 (MCP-1) in IA patients was 2.8-fold higher than healthy controls<sup>6</sup>. In addition, MCP-1 has a moderating effect on macrophage infiltration<sup>7</sup>; Matrix metalloproteinases are a family of endopeptidases that mediate vascular remodeling by degrading extracellular matrix components, such as collagen and

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elastin. Compelling evidence implicates increased amounts MMPs activity in the pathogenesis of aneurysms, MMP-2 and MMP-9<sup>8-10</sup> are commonly reported. We explored the early expression of NF- $\kappa$ B, MCP-1 and -MMP-9 in a rabbit carotid aneurysm model and investigated the possible mechanisms of aneurysm.

## MATERIAL AND METHODS

### Material

Porcine pancreatic elastase (active unit of 4 U/Mg) was purchased from Sigma company (Tex, USA); ABC immunohistochemistry Kit was purchased from Huamei Biotech Companies (Shanghai, China); Mouse anti rabbit NF- $\kappa$ B, MCP-1 and MMP-9 immunohistochemical polyclonal antibodies were purchased from Abcam (MA, USA); RNAlater solution and Trizol were purchased from Invitrogen (NY, USA); Fluorescence quantitative PCR primers were synthesized by Shenggong Biological Technology Co., Ltd. (Shanghai, China); Fluorescence quantitative PCR detection system was supplied by Bio-Rad (PA, USA).

All experimental procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of First Affiliated Hospital of Suzhou University. All efforts were made to ameliorate suffering of animals.

### Intracranial aneurysm induction

Twenty four adult New Zealand rabbits (2400-2600g) aged from five to six months, were purchased from the animal center of Suzhou University, and were divided into four groups: normal control (Group A); Rabbits received induction for 1 week (Group B); Rabbits received induction for two weeks (Group C); Rabbits received induction for three weeks (Group D).

A total of 3.5 ml/kg 10% chloral hydrate were injected intraperitoneal for anesthesia. The right common carotid artery (CCA) was exposed and operated on under microscope. A 5-10 mm diameter CCA near the right external carotid artery bifurcation was measured by a vernier caliper, the partial fibrous connective tissue of the surface was gently torn, then infused with 0.2 ml of elastase (EA, 50 / ml) to the surface, using a micropipet. Twenty minutes (min) later, 500 U heparin sodium was injected to the rabbit ear intravenously. Phosphate buffered saline was infused in the control group.

### RT-PCR analysis

After the rabbits were given a general anaesthetic along the neck incision, we exposed the right common carotid artery. A vernier caliper was used to measure the size (width, height) of neck aneurysms. The aneurysm specimens were cut into pieces. Fifty to 100 mg homogenate was prepared and 1 ml Trizol reagent was used to extract the total RNA. Extracted RNA concentration was measured by nucleic acid extermimator. The full-length rabbit mRNA sequence was searched from Gene Bank; fluorescence quantitative PCR primers were synthesized by Shanghai SANGON Biological Technology Co., Ltd. The upstream primer sequence of NF  $\kappa$ B was: 5'CGC ATCCAG ACC from the C C A-3', R: 5' TGC CCAGAA: a GGA AAC ACC A 3'. MCP 1, F: 5'-ATC TCA GTG AAG AGG CTA ATG-3' and R: 5'- GTG TYG GGT TGT GGA - 3'; MMP9, F: 5'-TGT GTC TTC CCC TTC GTC TTC C-3', R: 5'-GCC CCA CTT CTT GTC GCT GT-3'.

### Histological and immunohistochemical assessment

Resected specimens of aneurysm were fixed in 4% neutral formaldehyde solution. After dehydration for 24 hours (h), the tissue was embedded in paraffin and specimens were cut into serial sections. Routine hematoxylin-eosin (HE) staining and immunohistochemical examination were conducted. For HE staining, the paraffin sections were stained by hematoxylin eosin and examined under light microscope.

SABC method was used for immunohistochemical staining, 0.3% methanol hydrogen peroxide was used to inactivate endogenous peroxidase for 30 min at room temperature. Samples were washed using PBS (0.1 mol/L) three times, digested by complex digestive solution for 10 min at room temperature, washed by PBS three times, then blocked normal goat serum solution for 20 min. Mouse anti rabbit NF- $\kappa$ B, MCP-1 and MMP 9 antibody (with concentration of 1:200) were applied respectively, cultured at 40°C overnight; Ig G (1:50) served as the second antibody at 37°C for 30 min; SABC served as third antibody at 37°C for 20 min; coloured by DAB. After dehydration and xylene, sections were observed under the microscope. In control group, PBS served as negative control.

### Data expression and analysis

Immunohistochemistry results were obtained using Image Pro Plus 6 software using their absorbance (density) value. RT-

**Table 1: mRNA expression of NF- $\kappa$ B, MCP-1 and MMP-9 in each group ( $2^{-\Delta C_T}$ )**

	Group A (N=6)		Group B (N=6)		Group C (N=6)		Group D (N=6)	
NF- $\kappa$ B ( $10^2$ )	0.2002	0.0762	9.7896	2.8993**	6.8706	4.7444*	1.8733	1.1556
MCP-1( $10^2$ )	0.8357	0.4307	15.8003	13.2745**	10.0268	4.0151**	6.8052	2.0054
MMP-9( $10^2$ )	0.9170	1.5298	10.4293	7.2007*	25.7293	12.3473*	32.9431	1.7531*

\*, P<0.05; \*\*, P<0.01, compared with group A.

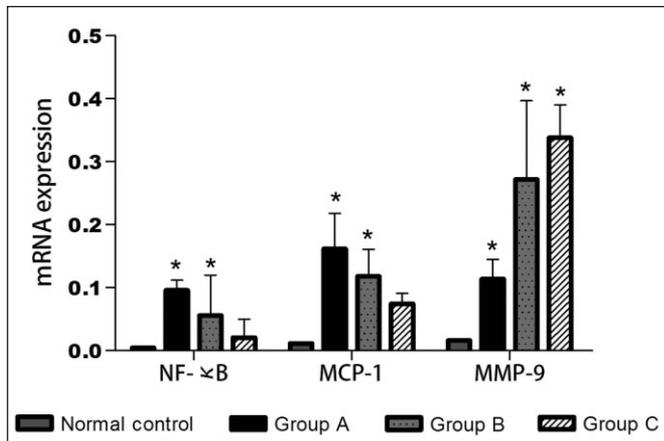


Figure 1: Column chart comparison of the mRNA expression of NF-κB, MCP-1 and MMP-9 in each group.

PCR results were expressed as  $2^{-\Delta C(T)}$ . Results were expressed as mean  $\pm$ SD. Data were analysed by SPSS 16.0 software. A significant difference was considered for  $P < 0.05$ .

**RESULTS**

**RT-PCR result**

Nuclear factor-κB were highly expressed in group B and group C but none in group D ( $P = 0.008, 0.041$  and  $0.558$

respectively when compared with group A); the expression have no statistical difference between group B and group C ( $P=0.317$ ). We observed a similar expression tendency of MCP-1 ( $P=0.001, 0.006$  and  $0.0558$  in group B, C and D respectively when compared with group A); the expression showed no statistical difference between group B and group C ( $P=0.124$ ); the expression of MMP-9 increased gradually, the difference in these group was statistically significant ( $P < 0.05$ ) (Table 1, Figure 1).

**Observation result of CTA aneurysms changing**

The six rabbits in the normal control group were sacrificed and CTA performed. The thickness of left and right arteries in the neck were equal. After one week induction with elastase I, aneurysm models were induced successfully in the 18 rabbit experimental groups. There were 7 cases of cystic artery aneurysm and 11 cases of fusiform aneurysm induced (Figure 2).

The aneurysm size in group B, C and D increased significantly compared with group A when observed by microscope (Table 2). The difference between these groups were significant ( $P < 0.05$ ). The height and width of the aneurysm in group D had no significant difference compared with group C. This demonstrated that elastase destroyed the right common carotid artery elastic fiber tube wall. The constant impact of blood flow gradually induced secondary generation of aneurysms and stable growth was maintained for two weeks.

**Table 2: Induced aneurysm size in each group**

	Group A	Group B	Group C	Group D
Aneurysm width (mm)	0	2.827 0.296	4.443 0.727*#	4.870 0.727*#
Aneurysm height (mm)	2.432 0.0422	3.712 0.303	5.580 1.432*#	6.140 1.488*#

\*,  $P < 0.05$ , compared with group A (normal control); #,  $P < 0.05$  compared with group B.

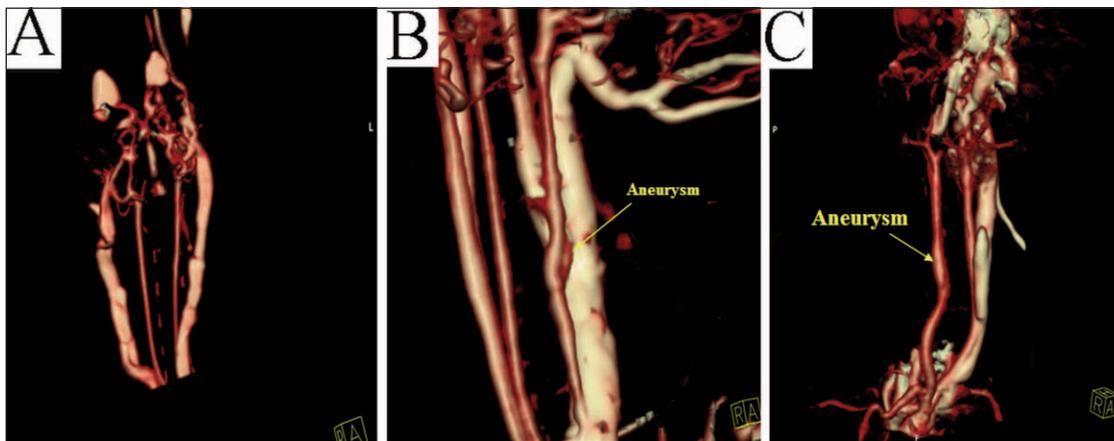
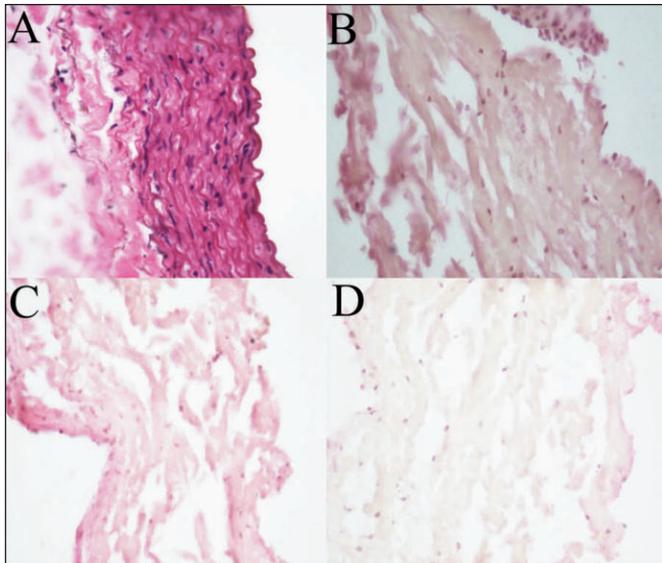


Figure 2: CT angiography of rabbit carotid artery. (A) Normal carotid artery; (B) lateral aneurysm induced for a week by elastase at the middle of right carotid artery (Saccular aneurysm, arrow); (C) An aneurysm induced for a week (Fusiform aneurysm, arrow).



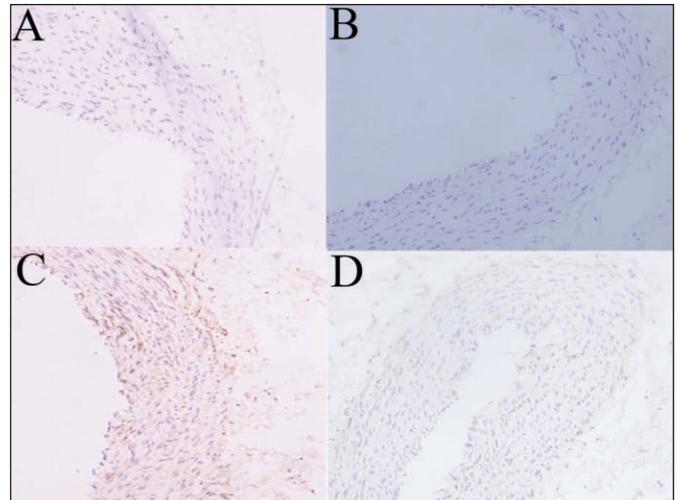
**Figure 3:** HE stains result. (A), normal control group, the vascular wall cells are tightly connected with intact elastic fiber structure. After induction, the number of smooth muscle reduced, endothelial cells were damaged; the aneurysm wall elastic layer was damaged more seriously, (B) induction for 1 week; (C) induction for 2 weeks; (D) induction for 3 weeks.

### HE staining results

After staining, we observed the positive expression of brown nucleus or cytoplasm. The observed aneurysm wall elastic fiber fractures were visible in the induced groups and the number of smooth muscle cells were reduced. Endothelial cells were damaged; the aneurysm wall elastic layer was damaged more seriously, and the number of smooth muscle cells decreased. We observed the thinnest aneurysm wall film at the third week induction group, which had only two to three cell layers. (Figure 3)

### Expression of NF- $\kappa$ B reaching peak at week 1

We observed positive expression of NF- $\kappa$ B (brown) existed in cytoplasm of vascular endothelial cells of the aneurysm wall, smooth muscle cells and inflammatory cells (Figure 4). Besides that, we observed highly expressed NF- $\kappa$ B in partial cells, high expression of NF- $\kappa$ B could be seen in nuclear. The endothelial



**Figure 4:** NF- $\kappa$ B expressions in each group. (A), normal control; (B), the expression of NF- $\kappa$ B reached a peak at week 1, and then decreased in week 2 (C) and week 3 (D).

cells and smooth muscle cells decreased gradually with increased damage to the elastic layer of the wall of the aneurysm. We observed elasticity fibre rupture in aneurysm walls in group D. Compared with the control group, the expression of NF- $\kappa$ B increased after induction, reaching a peak at week 1, and then decreased; the expression in group B and C having a significant difference ( $P < 0.001$ ,  $P < 0.001$ , respectively) when compared with control. Although we observed relative highly expression in group D, the expression had no differences between group A and group D (Table 3).

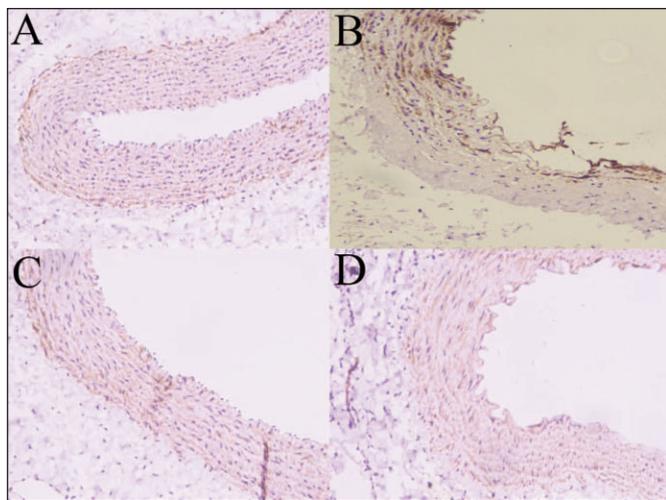
### Expression of MCP-1 reaching peak at week 1

Immunohistochemistry results showed positive visible MCP-1 expressed in cytoplasm of aneurysm wall endothelial cells, smooth muscle cells and inflammatory cells (Figure 5). We observed highly expressed MCP-1 in partial cells, high expression of MCP-1 could be seen in nuclear with decreased smooth muscle cells, and the aneurysm wall was damaged seriously. The cells thinned gradually with fuzzy hierarchical cell boundary. The expression of MCP-1 increased after induction, reaching a peak at week 1, and then decreased. The expression in the induction groups had statistical difference

**Table 3: Protein expression of NF- $\kappa$ B, MCP-1 and MMP-9 in each group**

	Group A (N=6)		Group B (N=6)		Group C (N=6)		Group D (N=6)	
NF- $\kappa$ B	0		0.0076	0.00478**	0.0066	0.00242**	0.0015	0.00101
MCP-1	0		0.0085	0.00194*	0.0074	0.00347*	0.0033	0.0014*
MMP-9	0.0002	0.00009	0.0132	0.0052*	0.0307	0.00548*	0.0489	0.00694*

Note: the unit is absorbance density value; \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ , compared with group A.



**Figure 5:** The expression of MCP-1. (A), normal control; (B), the expression increased after induction, reaching a peak at week 1 (B), and then decreased in week 2 (C) and week 3 (D).

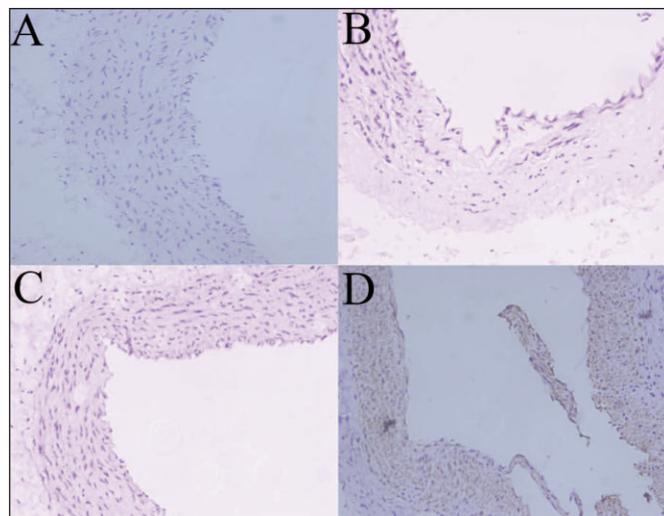
when compared with control ( $P < 0.05$ ), but the expressions have no difference between group B and group C ( $P = 0.367$ ).

#### Elevated MMP-9 expression from group A to group D

From group A to group D, we observed increased MMP-9 expression. The expression between the four groups showed statistical difference ( $P < 0.05$ ). Positive expression of MMP-9 could be seen in the endothelial cells of the aneurysm wall and in mid-membrane smooth cells. The nuclei stained blue and yellow and nuclei fusion could be observed. The cytoplasm was brown and the inner elastic membrane disappeared gradually. The mid-membrane smooth muscle cells decreased, the elastic layers of the aneurysm were damaged further and ultimately fractured (Figure 6).

#### DISCUSSION

In recent years, more and more research has indicated that the inflammatory reaction and extracellular enzymes are involved in the process of aneurysm formation<sup>11-13</sup>. Kosierkiewicz *et al*<sup>14</sup> found that inflammatory reaction was present in unruptured and ruptured cells of aneurysm walls. He concluded that inflammatory reaction exists before aneurysm rupture occurred. The aggregation of inflammatory cells and excessive degradation of the extracellular matrix outside cells is a typical feature of intracranial aneurysm. Chronic inflammatory reaction and pathological remodeling of extracellular matrix in the aneurysm wall may contribute to the occurrence and development of aneurysm<sup>2,6</sup>. In essence, the formation, growth and rupture of aneurysm is the process of vascular remodeling due to inflammatory reaction caused by various factors. Compelling studies indicate that NF- $\kappa$ B terminal is one of the pivotal factors contributing to the expression of a pro-inflammatory gene, resting state NF- $\kappa$ B existed in the cytoplasm as inactive form. When stimulated by multifactor, NF- $\kappa$ B is



**Figure 6:** The expression of MMP-9 was relatively low in normal control (A); and increased from group B (B), group C (C) to group D (D) after induction. Positively expressed MMP-9 could be seen in the endothelial cells of the aneurysm wall and in mid-membrane smooth cells.

released by cells transduction signal, then vectored by the kernel boot sequence into the nucleus. NF- $\kappa$ B starts to regulate the expression of downstream target genes<sup>15,16</sup>. Studies have shown that NF- $\kappa$ B could induce the expression of chemokines, adhesion factor, MMPS and so on<sup>17-19</sup>. These factors play a direct regulatory role in the activation, proliferation, invasion, migration and secretion in corresponding cell.

In this study, we found that the expression of NF- $\kappa$ B is enhanced in the early development of cerebral aneurysm. NF- $\kappa$ B mainly expressed in endothelial cells, smooth muscle cells, and inflammatory cells, and mainly localized in the cytoplasm. We observed strong positive expression in the nuclei of aneurysm cells. This observation confirmed aneurysm is in the activated state. We speculate that NF- $\kappa$ B plays an important role in aneurysm wall inflammation and vascular pathology of cerebral aneurysms. Activation of NF- $\kappa$ B may be one of the initiating factors attributing to the occurrence and development of cerebral aneurysm.

The infiltrations are closely related to the injury of elastic fibers and collagen fibrils and macrophages are an important component of inflammatory cells. MCP-1, an important single edge macrophage chemotactic factor, could regulate the expression of adhesion molecules  $\alpha$ -4, integrin  $\beta$ -2, and leukocyte adhesion molecule-1 on cell surface, thus regulating a variety of adhesion molecules by membrane binding activity promoting monocyte accumulation in the lesion area, gradually making the artery show aneurysmal dilatation by releasing a variety of molecules (such as MMPs), which could degrade elastic fibers, collagen fibers in arterial wall<sup>20,21</sup>. In our study, we found that MCP-1 was highly expressed in the cerebral aneurysms model. MCP-1 was mainly expressed in smooth muscle cells and endothelial cells, suggesting that the aneurysm wall could produce chemokines continually, thus strengthening the inflammatory response. mRNA expression of MCP-1 was

fairly low in normal arteries, but reached a peak after induction for one week, demonstrating that the expression of MCP 1 could be used as an early indicator for detection of cerebral aneurysms. Along with the sustained induction, the damaged arterial wall elastic fiber is gradually increased. We observed more significant dilatation of the aneurysm artery. As one of the early expressed molecules, the products of MCP-1 induced macrophage adhesion and infiltration in the artery wall of cerebral aneurysms, further promoting the degradation of elasticity fiber in the artery wall, and contributed to the occurrence and development of the brain aneurysm.

Aoki *et al* proposed that MMPs, especially MMP-9, promote decreased blood vessel wall tension, which leads to the formation of aneurysms<sup>22</sup>. The study showed their expression is elevated in abdominal aortic aneurysm<sup>23,24</sup>. This leads to the balance broken between MMP-9 and TIMP-2, and plays an important role in the formation of aneurysms. Few studies involved the change of MMPs during the early stage of cerebral aneurysm. We demonstrated that the elastic fibers of extracellular matrix structure in experimental cerebral aneurysms were damaged. Elastic fibers decreased or disappeared with a large number of infiltrated inflammatory cells. The expression of MMP-9 was obviously enhanced in the aneurysmal wall; positive expression of MMP-9 could be observed throughout the aneurysmal wall. These results indicate that the damage of elastic fibers is one of the key factors for aneurysm formation; increased infiltration of inflammatory cells and the secretion of MMP-9 are the main reasons that caused elastic fiber damage. Generally speaking, MMP-9 is not expressed in normal blood vessels. This is not consistent with our findings. We concluded it was due to the young age of rabbit, active metabolism of matrix and existed cross reaction in polyclonal antibody.

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Yanfei Liu\* and Yongqiang Zhang\* contributed equally to this manuscript.

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