

Original Article

Anti-inflammatory effects of artificial synthetic E-selectin on common carotid artery aneurysm in an elastase induced rabbit model

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Abstract: In the present study, we tested the effects of artificial synthetic E-selectin on expressions of NF- κ B and MCP-1 in an elastase-induced rabbit model of common carotid artery aneurysm. 42 New Zealand rabbits (21 male and 21 female animals) were randomly divided into three (control, disease, and treatment) groups. The right common carotid artery was exposed and injected with either 0.2 ml of phosphate-buffered saline (control group) or elastase (disease and treatment groups). The rabbits in the treatment group received artificial synthetic E-selectin (1 mg/kg/day for up to 3 weeks) through a venous catheter. Morphologic changes of the right common carotid artery and aneurysm formation were assessed by CT angiography and hematoxylin & eosin staining. Specimens of aneurysm tissue were collected, and mRNA and protein expressions of NF- κ B and MCP-1 were evaluated by, respectively, qPCR and immunohistochemistry. Compared with the disease group, animals treated with artificial synthetic E-selectin showed decreased dimensions of aneurysms, with the differences becoming significant starting from week 2 ($P < 0.05$). Furthermore, expressions of NF- κ B and MCP-1 (both mRNA and protein) were decreased in treated animals. Artificial synthetic E-selectin inhibits expression of NF- κ B and MCP-1 in an experimental model of common carotid artery aneurysm. Treatment with artificial synthetic E-selectin may attenuate aneurysm development.

Keywords: Aneurysm, MCP-1, model, NF- κ B, artificial synthetic E-selectin

Introduction

An intracranial aneurysm rupture is the major cause of subarachnoid hemorrhage. It is characterized by rapid onset, and high mortality and disability rates. Since introduction of microneurosurgery and neurological interventions, mortality and disability rates decreased substantially. However, the overall prognosis still remains unfavorable. This is mainly due to an incomplete understanding of occurrence, development, and rupture of intracranial aneurysm [1, 2]. In recent years, prominent role of inflammation in the development of intracranial aneurysm was demonstrated by numerous studies. Yet, only few studies evaluated the efficacy of anti-inflammatory therapies for intracranial aneurysm. In the present study, we tested potential anti-inflammatory effects of artificial synthetic E-selectin on expression of the inflammatory factors Nuclear Factor (NF)- κ B and

monocyte chemotactic protein-1 (MCP-1) in an elastase-induced rabbit model of common carotid artery aneurysm.

Materials and methods

Reagents

Porcine pancreatic elastase was from Sigma-Aldrich (St. Louis, MO, USA). The ABC immunohistochemistry kit was purchased from Xinpeng Hongye Technology (Beijing, China). Mouse anti-rabbit NF- κ B and MCP-1 polyclonal antibodies were from Abcam (Cambridge, UK), RNA lysis solution was supplied by Applied Biosystems (CA, USA), while RNA extraction Trizol reagent was from Invitrogen (Carlsbad, USA). The primers for qPCR were synthesized by Biological Technology (Shanghai, China). Synthetic E-selectin was supplied by Shanghai Institute of Biochemistry (Shanghai, China).

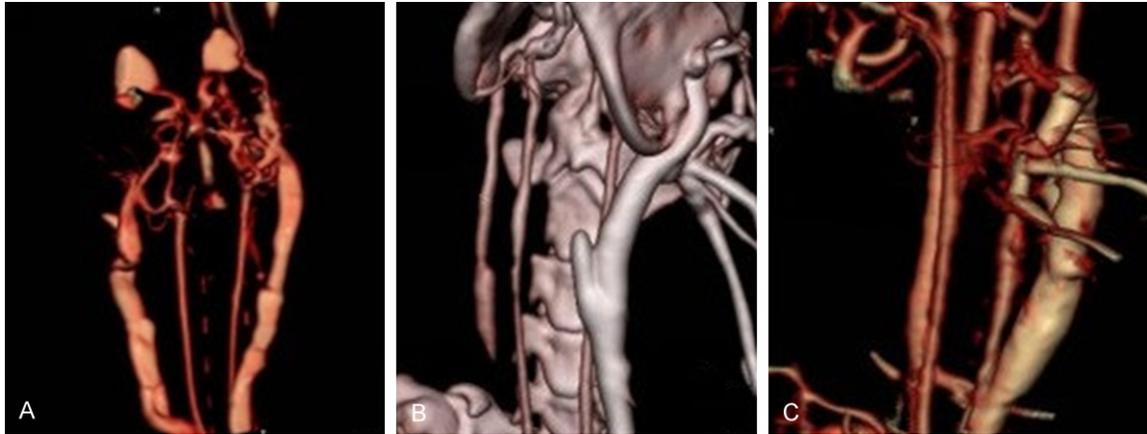


Figure 1. CTA findings. Aneurysms in control (A), disease (B), and treatment (C) groups. The animals in the latter group received synthetic E-selectin for one week.

Table 1. The mean size of aneurysms

| Group | Cases | Width (mm) | Height (mm) |
|-------|-------|----------------|----------------|
| A | 6 | 0 | |
| B | 6 | 2.4784±0.3155 | 2.9137±0.2922 |
| C | 6 | 4.1412±0.7169 | 4.9230±1.3295 |
| D | 6 | 4.5620±0.7111 | 5.3400±1.5033 |
| E | 6 | 2.3822±0.4333 | 2.8584±0.3587 |
| F | 6 | 2.3216±0.8356* | 2.8841±1.4895* |
| G | 6 | 2.4084±0.5451# | 2.9642±1.0519# |

Note: *P < 0.05 vs. group C; #P < 0.05 vs. group D.

Experimental animals

The animal use and experimental design were approved by the local Animal Ethics Committee. Forty-two New Zealand white rabbits were supplied by the Experimental Animal Center of Suzhou University. There were 21 male and 21 female animals, weighing 2.5-3 kg and aged 5-6 months. The animals were randomized into three groups: control (6 animals), disease (18 animals), and treatment (18 animals) groups. Both disease and treatment groups were divided into three subgroups which were followed for one, two, or three weeks. Each of these subgroups comprised of 6 animals.

Animal model and artificial synthetic E-selectin

Under intraperitoneal anesthesia with 10% chloral hydrate (3.5 ml/kg), the right common carotid artery was exposed under microscopic view. Superficial fibrous connective tissue was removed around the right common carotid artery segment at 0.5-1.0 cm proximally to the

bifurcation between the internal and external right carotid arteries. The segment of the vessel at the administration site was depressed using a gentle pressure to maintain its relatively inferior position. The animals in the disease group received an injection of 0.2 ml of elastase. Animals in control group received phosphate-buffered saline instead of elastase. Animals in the treatment group received an injection of 0.2 ml of elastase, as well as artificial synthetic E-selectin (1 mg/kg/day) administered through venous catheter simultaneously with heparin infusion. In all animals, a 24 G intravenous catheter was inserted into the right ear vein after the injection. Through the catheter, 500 U of sodium heparin were slowly infused. The rabbits in the disease and treatment groups were euthanized on days 7, 14, and 21.

Specimens

After the anesthesia, the right common carotid artery was exposed along the original incision site on the neck. The width and height of the right common carotid artery aneurysm were measured using vernier caliper. Aneurysms were removed and tissues sampled. Some specimens were fixed for 24 hours with 4% neutral formalin and further processed by dehydration, xylene treatment, paraffin embedding, and sectioning (8-µm slices). The slices were stained with hematoxylin and eosin staining for light microscopy or used in immunohistochemistry. Parallel slices were treated with RNA later and kept at -20°C for subsequent RNA isolation, reverse transcription, and qPCR.

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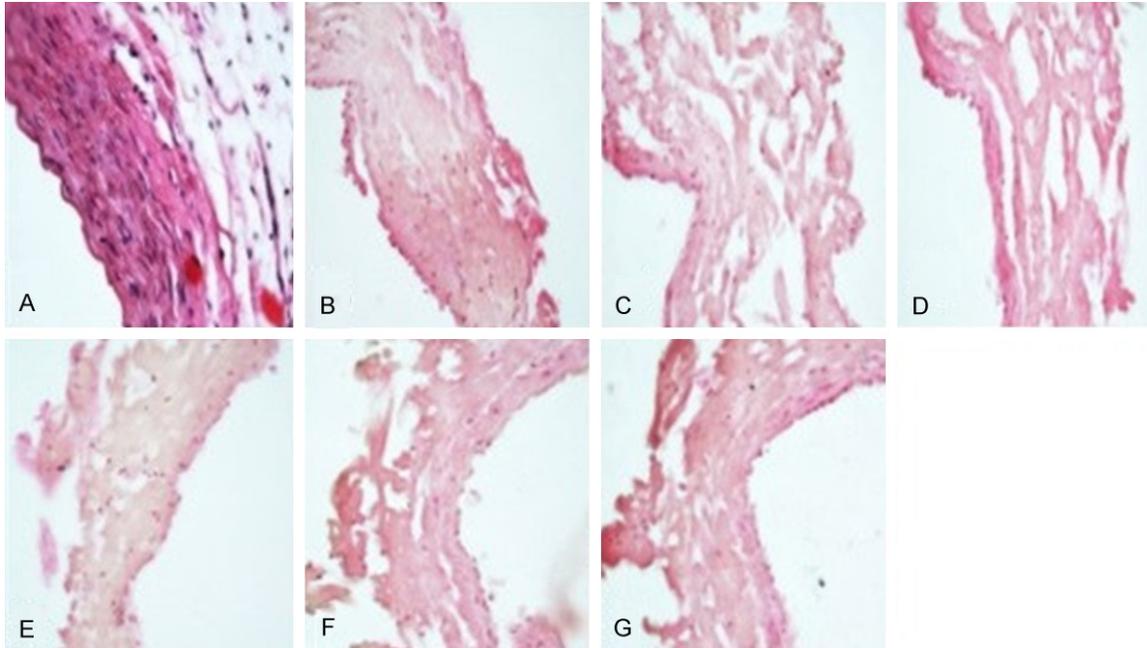


Figure 2. Hematoxylin & eosin staining. A. Control group; B-D. Disease group at weeks 1, 2, and 3; E-G. Treatment group after application of synthetic E-selectin for 1, 2, or 3 weeks. Magnification $\times 200$.

Table 2. The expressions of NF- κ B and MCP-1 protein (mean density)

| Group | Cases | NF- κ B | MCP-1 |
|-------|-------|------------------------|------------------------|
| A | 6 | 0 | 0 |
| B | 6 | 0.0075 \pm 0.0042 | 0.0084 \pm 0.0020 |
| C | 6 | 0.0064 \pm 0.0037 | 0.0073 \pm 0.0032 |
| D | 6 | 0.0023 \pm 0.0020 | 0.0033 \pm 0.0023 |
| E | 6 | 0.0073 \pm 0.0025 | 0.0082 \pm 0.0032 |
| F | 6 | 0.0049 \pm 0.0015* | 0.0051 \pm 0.0025* |
| G | 6 | 0.0010 \pm 0.0009#,& | 0.0018 \pm 0.0016#,& |

Note: *P < 0.05 vs. group C; #P < 0.05 vs. group D; &P < 0.05 vs. group F.

Computed tomographic angiography (CTA)

CTA examinations were done to evaluate common carotid arteries pre-operatively and on post-operative day 7. Local morphological changes and formation of carotid artery aneurysms were examined at the site of elastase application.

Immunohistochemistry

Endogenous peroxidase was inactivated by 0.3% H₂O₂ (30 min, room temperature). Samples were further treated with polyanzyme digestive solution (10 min, room temperature).

The slides were then washed three times with phosphate-buffered saline. Afterwards, the slides were blocked for 20 min with goat serum. Excessive serum was removed by centrifugation. Then, primary antibodies against NF- κ B or MCP-1 were applied at a 1:200 dilution, and slides were incubated overnight at 4°C. Afterwards, secondary antibody was added to slides at a 1:50 dilution and incubated for 20 min at 37°C. Staining was visualized according to the manufacturer's instructions for ABC kit. Positive staining was defined as brown yellow staining in the cytoplasm or nucleus, and compared with negative control slides in which primary antibody was omitted.

The optical density analysis was conducted with the help of Image-Pro Plus 6.0 software (Media Cybernetics, Maryland, USA). The results were presented as mean density. The area between measurement region and integrated optical density of target staining zone of this region were measured, and the ratio of these two values was taken as mean density.

Expression of NF- κ B and MCP-1 mRNA

Aneurysms was removed and pulverized, and 50-100 mg of tissue specimens was solubilized in 1 ml of Trizol reagent. Total RNA was extract-

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Table 3. The expressions of NF- κ B and MCP-1 mRNA ($2^{-\Delta\Delta Ct}$)

| Group | Cases | NF- κ B | MCP-1 |
|-------|-------|-----------------------------------|-----------------------------------|
| A | 6 | 0.0020 \pm 0.0008 | 0.0079 \pm 0.0059 |
| B | 6 | 0.0999 \pm 0.0418* | 0.1602 \pm 0.0959* |
| C | 6 | 0.0700 \pm 0.0433# | 0.1187 \pm 0.0396# |
| D | 6 | 0.0193 \pm 0.0102 | 0.0673 \pm 0.0212 |
| E | 6 | 0.0978 \pm 0.0373 | 0.1501 \pm 0.0596 |
| F | 6 | 0.0451 \pm 0.0345\$ | 0.0652 \pm 0.0215\$ |
| G | 6 | 0.0086 \pm 0.0125 $\&$ Δ | 0.0141 \pm 0.0093 $\&$ Δ |

Note: * $P < 0.05$ vs. group A; # $P < 0.05$ vs. group A; \$ $P < 0.05$ vs. group C; & $P < 0.05$ vs. group D; $\Delta P < 0.05$ vs. group F.

ed as per usual protocol. The following primer sequences were used in PCR reaction: NF- κ B forward primer 5'-CGCATCCAGACCAACAACA-3' and reverse primer 5'-TGCCCAGAAGGAAACA-CCA-3', and MCP-1 forward primer 5'-ATCTCA-GTGAAGAGGCTAATG-3' and reverse primer 5'-GTGTTCTTGGGTTGTGGA-3'. As endogenous control, 18S RNA was used (forward primer 5'-CCTGGATACCGCAGCTAGGA-3' and reverse primer 5'-CGCCGCGTTATGCTTACGGGG-3'). Conditions for qPCR amplification were as follows: 94°C for 2 min, 94°C for 15 sec, 60°C for 40 sec, 40 cycles. qPCR results were compared using the $2^{-\Delta\Delta Ct}$ method, where ΔCt is $Ct_{\text{target gene}} - Ct_{\text{endogenous control}}$.

Statistical analysis

Statistical analyses were carried out using the statistical software SPSS16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean \pm SD. LSD or ANOVA analyses were used to compare study groups. The p value of < 0.05 was considered to be statistically significant.

Results

Aneurysm sizes

In control group, CTA examination demonstrated similar sizes of the left and right common carotid arteries (**Figure 1A**). By contrast, in disease group, we observed aneurysms with the width and height of, respectively, 2-6 mm and 3-7 mm at the elastase application site (**Figure 1B**). There were no time-associated changes in the size of aneurysms. In the treatment group, application of artificial synthetic E-selectin for one week was associated with fusiform or saccular aneurysm formation (**Figure 1C**). Compa-

red with disease group, treated animals showed decreased dimensions of aneurysms, with the differences becoming significant starting from week 2 ($P < 0.05$; **Table 1; Figure 1C**).

Hematoxylin & eosin staining

No abnormal morphological findings were present in control group animals. By contrast, animals in disease group exhibited ruptures of elastic fiber, reductions of smooth muscle cells, and destruction of endothelial cells in aneurysms. These abnormalities worsened with time (**Figure 2**). For example, the thinnest aneurysms wall was observed in the animals that were exposed to the effects of elastase for 2 weeks. Specifically, these animals only had 2-3 layers in the tunica media (**Figure 2**).

In animals treated with artificial synthetic E-selectin for 1 week, decrease and atrophy of smooth muscle cells were also observed, as well as overt degenerative changes, aneurysm wall thinning, and rupture of elastic fiber (**Table 2; Figure 2**). However, as treatment continued, number of smooth muscle cells decreased, and there were less severe degenerative changes, slight thinning of the aneurysm wall, and mild rupture of elastic fiber (**Table 2; Figure 2**).

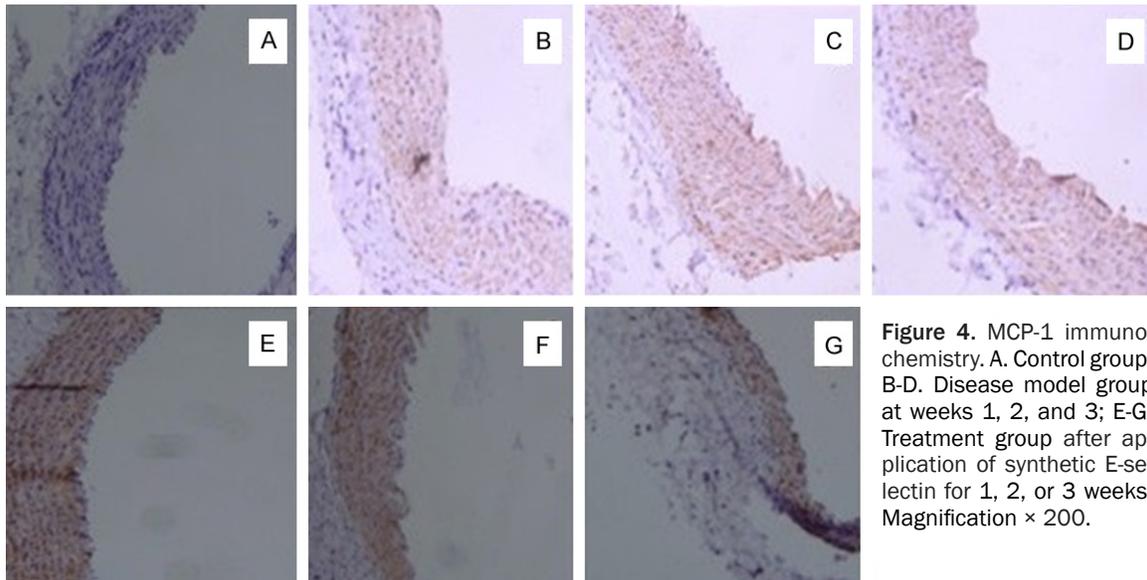
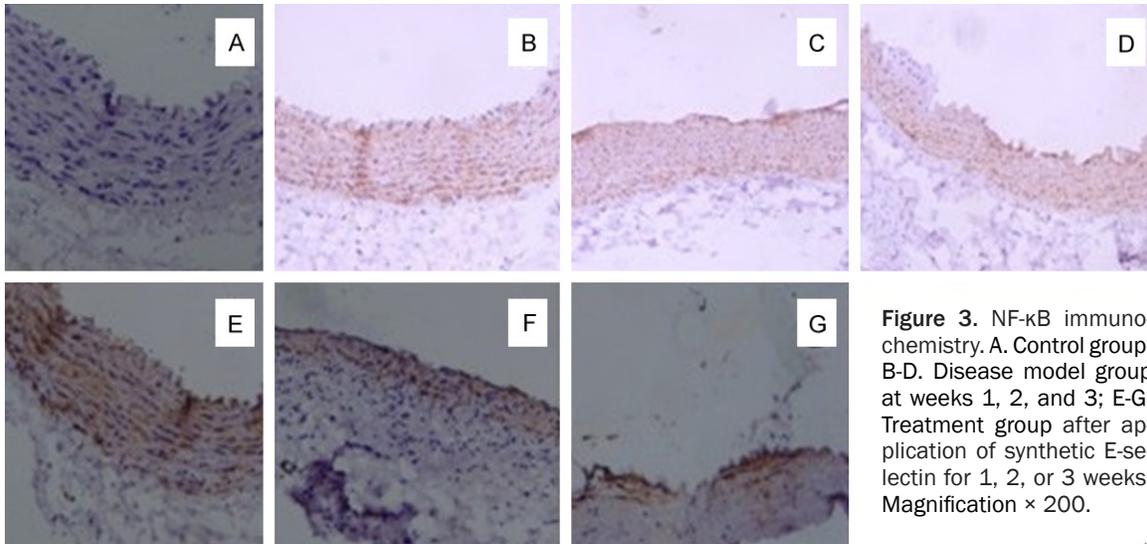
NF- κ B and MCP-1 expression (mRNA and protein)

NF- κ B expression was not observed in control group (**Table 2**). By contrast, animals in disease group overexpressed NF- κ B (**Table 2**). This expression did not depend on the duration of the aneurysm (**Table 2**). Treatment with artificial synthetic E-selectin decreased expression of NF- κ B (**Table 2**).

A similar trend was seen with expression of MCP-1: this protein was not expressed in control animals, was up-regulated in disease group, and was decreased by treatment (**Table 3; Figure 3**).

The trends observed at the protein level of both inflammatory factors were corroborated by studies of mRNA expression. Specifically, both NF- κ B and MCP-1 mRNAs were expressed at a very low level in control animals, while their expressions were up-regulated in animals of

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disease group. These expressions were suppressed by the treatment with artificial synthetic E-selectin (**Table 3; Figure 4**).

Discussion

Currently, surgical procedures, such as microsurgical clipping and endovascular coiling, are the only interventions believed to prevent the rupture of cerebral aneurysms. However, because of the risk of complications, surgical treatment is recommended only for a selected group of patients with an estimated high risk of rupture. Other patients with cerebral aneurysms are followed without effective treatment

and are at risk for rupture. Given the severity of arterial hemorrhage after the rupture of cerebral aneurysm, there is a strong demand for pharmacological treatment for cerebral aneurysm.

Selectins belong to the family of cell adhesion molecules and can be divided into three groups according to their expression sites: P-selectin (granule membrane protein, PADGEM, CD62P), E-selectin (endothelial-leukocyte adhesion molecule, ELAM, CD62E), and L-selectin (leukocyte selectin-1, LAM, CD62L). E-selectin is expressed on the surface of endothelial cells under basal conditions. Upon inflammatory condi-

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ons, endothelial cells further up-regulate E-selectin [3, 4]. E-selectin is responsible for identification, mediation and activation of leukocytes, as well as for stable leukocyte adhesion to endothelial cells and subsequent migration to extravascular tissues. Intranasal administration of E-selectin significantly attenuated cerebral ischemia-reperfusion injury in rats [5]. Artificial synthetic E-selectin is an oligopeptide based on the lectin fragment of selectin, with amino acids 23-30 from the N-terminal. The sequence is YTHLVAIQ. As demonstrated by previous *in vitro* studies, administration of artificial synthetic E-selectin is associated with suppression of E-, P- and L-selectin activities [6], interference of leukocyte adhesion to endothelial cells, reduction of migrating leukocytes and lymphocytes, and reduced or absent microvascular no-reflow phenomenon [7]. In animal studies, artificial synthetic E-selectin inhibits synthesis of inflammatory cytokines such as TNF- α or IL-6. In addition, artificial synthetic E-selectin is associated with suppression of excessive local inflammatory response of brain tissue following brain ischemia-reperfusion, elevation of oxygen partial pressure in the infarction area of brain tissues, and increase of cerebral blood flow, resulting in the improvement of oxygen supply in infarcted brain tissues and effective protection from brain ischemia-reperfusion [8].

Currently, there are no standard animal models for intracranial aneurysm. An aneurysm model induced by pancreatic elastase is considered to be quite representative of human cystic intracranial aneurysm both in pathology and hemodynamic characteristics [9, 10]. As demonstrated by the elastase infusion model of experimental aortic aneurysms of common carotid artery, development of aneurysms is associated with the phenotype conversion of smooth muscle cells of aneurysm walls from contractive type to synthetic type, and down-regulation of skeleton protein expression [11, 12].

Through recent experimental findings, we identified NF- κ B as a potential therapeutic target for cerebral aneurysms, inhibition of NF- κ B in animal models significantly suppresses the cerebral aneurysm formation, suggesting that anti-NF- κ B drugs can be utilized as therapeutic drugs for cerebral aneurysms. NF- κ B up-regulates expression of MCP-1 and other pro-inflam-

matory genes, and thus plays an important role in aneurysm formation. Expression of MCP-1 gene stimulates macrophage adhesion and aggregation to arterial walls, and release of matrix metalloproteinases that decompose elastic and collagen fibers of arterial walls, resulting in artery dilatation [13, 14]. NF- κ B activation is a potential initiating factor in the development and progress of cerebral aneurysm [15]. Based on this rationale, we chose to test synthetic E-selectin as a therapeutic in the aneurysm model. Expression of NF- κ B and MCP-1, and associated radiological and morphological changes were examined. We thus provided experimental evidence for establishment of a therapeutic against aneurysms.

Artificial synthetic E-selectin was demonstrated here to reduce the inflammatory response subsequent to arterial anastomosis. It also reduced proliferation of vascular smooth muscle cell and eliminated the stenosis of vascular anastomotic stricture. The mechanism is believed to involve a down-regulation of production of inflammatory cytokines such as TNF- α or IL-6 [16]. Therefore, administration of artificial synthetic E-selectin can lead to suppression of endothelial cells of the aneurysm, inhibition of leukocyte adhesion, reduction of circulatory leukocyte and lymphocyte numbers, inhibition of NF- κ B activation and MCP-1 expression, as well as inhibition of inflammatory response of the aneurysm cells, and prevention of aneurysm progression.

Conclusions

In the present study, we tested the effects of synthetic E-selectin on expressions of NF- κ B and MCP-1 in an elastase-induced rabbit model of common carotid artery aneurysm. Artificial synthetic E-selectin inhibits expression of NF- κ B and MCP-1 in this experimental model of common carotid artery aneurysm. We conclude that treatment with synthetic E-selectin may attenuate aneurysm development.

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Disclosure of conflict of interest

None.

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