

# Apolipoprotein E Expression in Experimentally Induced Intracranial Aneurysms of Rats

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**Objective :** An intracranial aneurysm is an important acquired cerebrovascular disease that can cause a catastrophic subarachnoid hemorrhage. Atherosclerosis is one of possible mechanism, but its contribution to aneurysm formation is unclear. Human apolipoprotein E (apoE) is best known for its arterial protection from atherosclerosis. In this study we observe apoE expression in experimental cerebral aneurysms of rats to elucidate the role of apoE in the process of cerebral aneurysm formation.

**Methods :** Twenty-four male 7-week-old Sprague-Dawley strain rats received a cerebral aneurysm induction procedure. One month (12) and three months (12) after the operation, the rats were killed, their cerebral arteries were dissected, and the regions of the bifurcation of the right anterior cerebral artery-olfactory artery (ACA-OA) bifurcations were examined histologically and immunohistochemically.

**Results :** In the 1 month group (n=12), the ACA-OA bifurcation showed no aneurysmal change in 7 rats and early aneurysmal change in 5 rats. In the 3 months group (n=12), the bifurcation showed no aneurysmal change in 2 rats and an advanced aneurysm in 10 rats. ApoE expression were in 3 specimen in early aneurysmal change, but not in advanced aneurysms.

**Conclusion :** ApoE expression in early aneurysmal wall suggests a possible role for apoE in early events leading to aneurysm formation. Further studies are necessary to elucidate the exact role of apoE in the pathophysiology of cerebral aneurysm.

**KEY WORDS :** Cerebral aneurysm · Atherosclerosis · Apolipoprotein E.

## Introduction

The mechanisms of intracranial berry aneurysm formation, growth, and rupture are complex. Inherent structural weaknesses of cerebral vessels, including the absence of an external elastic lamina and a unique branching pattern, together with pulsatile hemodynamic bombardment, lead to increase shear stresses at bifurcations<sup>7,27,29,30</sup>. Atherosclerosis may be present, but its contribution to berry aneurysm formation is unclear.

Many studies have been performed with the use of cerebral aneurysm specimens obtained during surgery or autopsy. However, these studies could not identify the key molecule for the development of cerebral aneurysm because the cerebral aneurysms used in the studies were always mature ones. Hashimoto et al developed a method to induce experimental cerebral aneurysms in rats without performing any direct manipulations of the cerebral artery itself<sup>10</sup>. Their experimental cerebral aneurysms

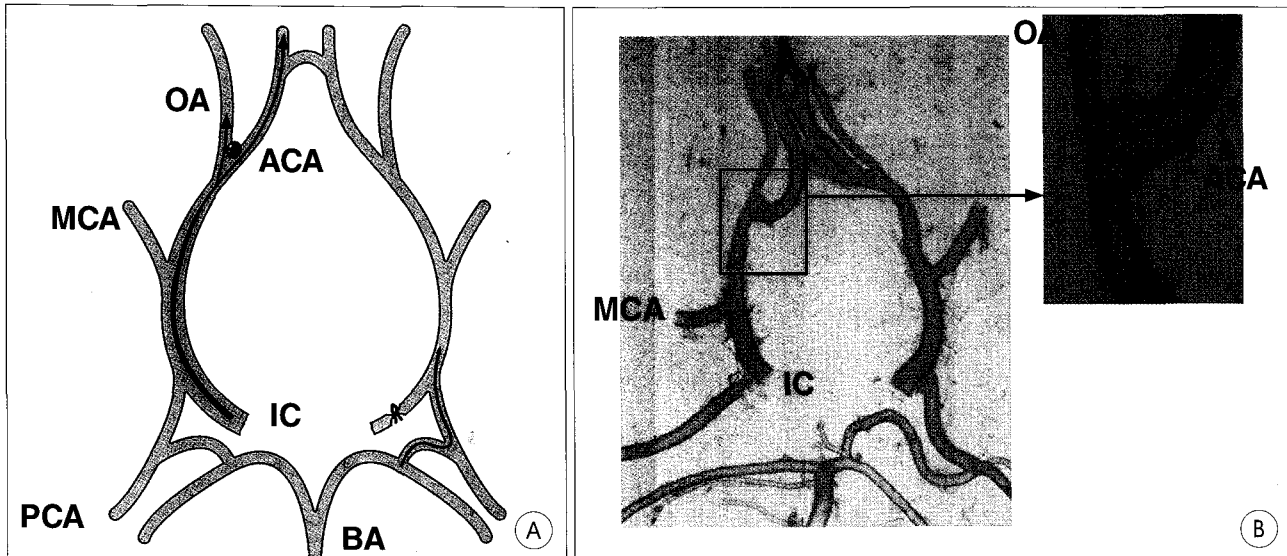
in rats resemble human cerebral aneurysms in their anatomic location and histological structure<sup>12</sup>. This model enables us to explore the process of cerebral aneurysmal development.

Apolipoprotein E (apoE), a 34,200-kd protein consisting of 299 amino acids, has a major role in the metabolism of lipids and lipoproteins<sup>17</sup>. ApoE also has a key role in the atherosclerotic process. The ApoE4 allele commonly is associated with an increased prevalence of coronary heart disease<sup>34</sup>, as well as of ischemic cerebrovascular disease, independently of plasma lipid levels<sup>18</sup>. ApoE not only influences plasma lipoprotein levels, but also facilitates cellular cholesterol efflux from foam cells, regulates the inflammatory process, and possesses antioxidant activity locally in the intima wall of vessels<sup>8</sup>.

In this study we applied the induction procedure of cerebral aneurysm to rats and observed the experimental cerebral aneurysms in early and advanced stage with the goal of clarifying the role of apoE in the process of cerebral aneurysm formation.

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**Fig. 1.** A : Schema of the procedure inducing an increase in hemodynamic stress at the right anterior cerebral artery–olfactory artery (ACA–OA) bifurcation, which is at the opposite side of the ligating carotid artery. B : Cerebral aneurysm is experimentally induced at right ACA–OA bifurcation in the circle of Willis in a Sprague–Dawley rat (arrowhead). MCA indicates middle cerebral artery; IC, internal carotid artery; BA, basilar artery; PCA, posterior cerebral artery.

## Materials and Methods

### Induction of experimental cerebral aneurysms

The left common carotid artery and posterior branches of both renal arteries of 24 male 7-week-old Sprague-Dawley strain rats each weighing 200 to 300g were ligated to induce cerebral aneurysms (Fig. 1). These procedures were performed with the rats under intraperitoneal sodium pentobarbital anesthesia (40mg/kg) with additional injections when necessary. After the operation, 1% saline was substituted for the drinking water to enhance the degree of hypertension. As a control, 12 age-matched, untreated male rats were used. Animal care and experiments complied with community standards on the care and use of laboratory animals.

Systolic blood pressure was measured twice by the tail-cuff plethysmographic method with rats in an unanesthetized state just before the operation and just before death.

### Tissue preparation and histopathology

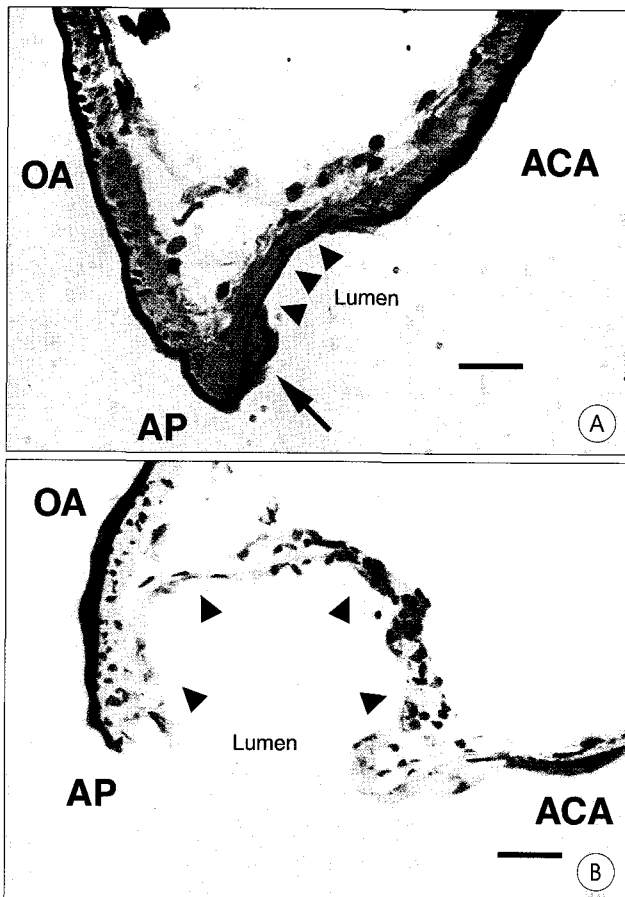
To obtain various degrees of aneurysms, we used two different periods of induction. One month after the start of the experiment, 12 of the 24 experimental rats were deeply anesthetized with ether and perfused transcardially with 0.1 mol/L PBS followed by 4% paraformaldehyde at a pressure of approximate 200mm H<sub>2</sub>O. Three months after the induction procedure, the remained rats were prepared by the previous method. Cerebral arteries were stripped from their brains under a surgical microscope. The right anterior cerebral artery–olfactory artery (ACA–OA) bifurcations, where aneurysmal changes of various degrees were speculated to be induced, were cut from the circles

of all rats. The each samples were embedded in OCT compound (Tissue-Tek), and 4- $\mu$ m thin sections were cut with a Leica CM 1850 and mounted on silane-coated slides. We could make 10 to 15 slides per 1 sample containing the aneurysm. Aneurysm, as defined here, refers to an outward bulging of the arterial wall detected by light microscopy. We defined the terms to describe the development of cerebral aneurysms as follows : Early cerebral aneurysm consists of discontinuity of the internal elastic lamina, which can be visualized by orcein stain, and no apparent outward bulging of the vascular wall. Advanced cerebral aneurysm refers to an obvious outward bulging of the arterial wall with complete disappearance of the internal elastic lamina (Fig. 2).

Three independent researchers assessed the histopathological changes in a blinded manner.

### ApoE immunohistochemistry

The sections were washed 3 times with 0.1 mol/L PBS for 5 minutes each time. After a section had been blocked with 5% normal donkey serum, goat anti-mouse ApoE antibody (1:400 dilution, Santa Cruz Biotechnology) was applied overnight at 4°C. Then the slides were washed 3 times with PBS and incubated with secondary antibody (anti-goat Alexa-Fluor 488 antibody, Molecular Probes) for 1 hour at room temperature. After they were washed 3 final times with PBS, the sections were covered and excited for fluorescence by illumination through a confocal laser scanning microscope system (FV300, Olympus). We performed the immunohistochemical staining in the same manner without the primary antibodies and confirmed that no signal was identified. We used rat lung,



**Fig. 2.** Various stages of cerebral aneurysms in light microscopic study (Orcein stain). A : Early stages, initial changes were localized almost exclusively at the intimal pad (arrow) and its neighboring distal portion. Arterial wall does not significantly protrude (arrowheads), but has the fragmentation of internal elastic lamina and slight thinning of the medial smooth muscle cell layer. B : Advanced stages. Arterial wall protrusion is clear and consists mainly of degenerated connective tissue (arrowheads). AP indicates apex; OA, olfactory artery; ACA, anterior cerebral artery. Scale bar = 20µm.

palatine tonsil, and aorta for positive controls of apoE and  $\alpha$ -smooth muscle actin, respectively.

### Double staining

Sections were first washed 3 times with PBS, and then diluted normal donkey serum was applied to them for blocking. The double staining was performed by using goat anti-mouse apoE antibody (Santa Cruz Biotechnology) and Cy3-conjugated mouse monoclonal anti- $\alpha$ -smooth muscle actin antibody (Sigma) as primary antibodies. The sections were incubated with the solution of primary antibodies overnight at 4°C. The slides were then washed 3 times with PBS and subsequently incubated with secondary antibody (anti-goat Alexa-Fluor 488 antibody and anti-mouse Alexa-Fluor 594 antibody, Molecular Probes) solution for 1 hour at room temperature. After they were washed 3 times with PBS, the sections were examined as described above.

### Statistical Analysis

The values of blood pressure were expressed as mean  $\pm$  SD. Statistical analysis was performed by using  $\chi^2$  test. Differences were considered statistically significant at  $P < 0.05$ .

## Results

### Blood pressure measurement

The systolic blood pressure of 1 month group just before operation was  $102.5 \pm 3.6$  mmHg, and that just before death was  $145.7 \pm 8.4$  mmHg, the difference being significant ( $P < 0.001$ ). The systolic blood pressure of 3 months group just before operation was  $104.3 \pm 5.4$  mmHg, and that just before death was  $167.7 \pm 11.3$  mmHg, the difference being significant ( $P < 0.001$ ). The systolic blood pressure of the 12 control rats ( $103.5 \pm 8.0$  mmHg and  $105.1 \pm 1.2$  mmHg, respectively) was not significantly different ( $P = 0.450$ ).

### Light microscopic study

In all 12 control rats, the wall of the ACA-OA bifurcations on both sides consisted of normal arterial components, ie, endothelial cells, internal elastic lamina, medial smooth muscle cells, and thin adventitia.

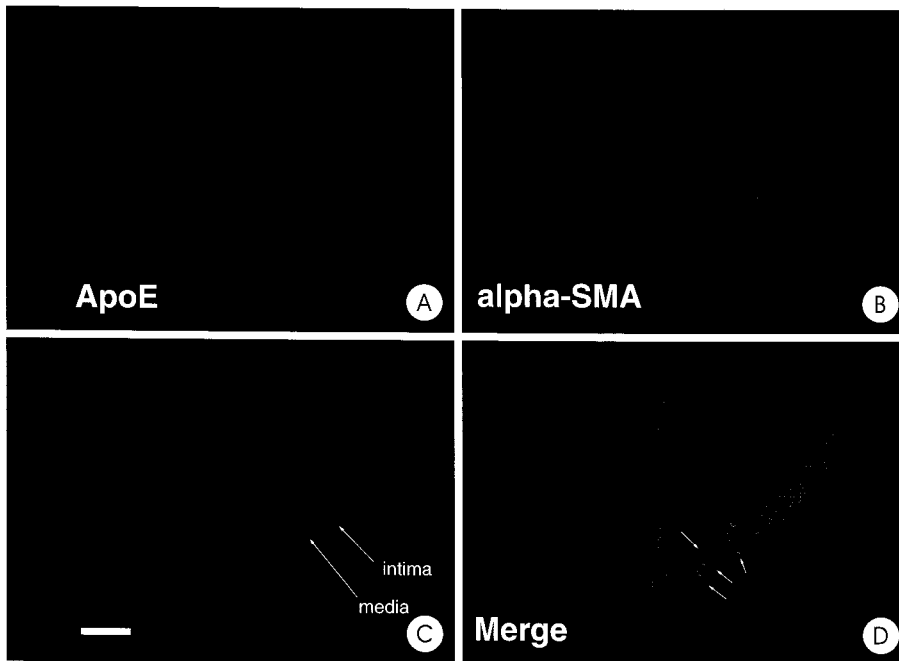
In 1 month group, the bifurcations showed no change in 7 rats, and early aneurysmal change in 5 rats. None of the bifurcations showed an advanced aneurysm. In 3 months group, the bifurcations showed no change in 2 rats, and advanced aneurysm in 10 rats. None of the bifurcations showed an early aneurysmal change.

The histological features of early aneurysmal changes were thinning of the medial smooth muscle layer accompanied by fragmentation of internal elastic lamina but no apparent outward bulging of the vascular wall. In the advanced aneurysm group, wall dilatation became apparent. In proportion to the dilatation size, the wall tended to become thinner. In the thinned parts of the wall, the decrease in smooth muscle cell number and disarrangement advanced. The degenerated changes of the internal elastic lamina tended to be advanced in proportion to the changes of the medial layer. The internal elastic lamina was discontinuous near the entrance of the lesion and nearly disappeared at the dome.

No aneurysmal changes were observed in the left ACA-OA bifurcations of any experimental rats. The findings of the walls were similar to those of the normal arterial wall, although they tended to be thicker.

### Immunohistochemistry of apoE

In 1 month group, 3 of 5 experimental cerebral aneurysms induced revealed strong immunoreaction indicating apoE, which was mainly observed in the media and adventitia (Fig. 3).



**Fig. 3.** Double staining. A : Green color represents apolipoprotein E (apoE), B : Red color represents  $\alpha$ -smooth muscle actin (alpha-SMA), a marker of vascular smooth muscle cells. C : Differential interference contrast image shows the vascular wall constituents. D : A+B. Yellow fluorescence indicates vascular smooth muscle cells affected by apoE. Bar=20 $\mu$ m.

We also performed apoE immunohistochemistry for all samples in 3 months group but that no apoE immunoreaction was observed (data not shown).

## Discussion

Development and rupture of cerebral aneurysm include a complex biological response reflecting the interplay of various inherited and acquired factors<sup>11,12</sup>. Genetic factors, hypertension, atherosclerosis, age, sex and smoking have also been reported to affect this disease<sup>19,21,22,26</sup>. In particular, hemodynamic stress has been shown in many investigations to be the major cause of various degenerative changes in cerebral aneurysm formation<sup>9,16,20,29,32</sup>. As for induced vascular remodeling of cerebral aneurysm, multiple mechanism such as ischemia of arterial walls<sup>25</sup>, endothelial injury<sup>15</sup> and disturbed extracellular matrix synthesis<sup>1</sup> have been found. Some molecular mechanisms such as deficiency of collagens (types III and IV)<sup>23,28</sup>, active expressions of matrix metalloproteinases<sup>4,14</sup> and apoptosis of medial smooth muscle cells<sup>16</sup> have been shown to be associated with cerebral aneurysm. Recently, Sadamasa et al noted that disruption of gene for inducible nitric oxide synthase (iNOS) reduces progression of cerebral aneurysm<sup>24</sup>. The mechanism of nitric oxide (NO)-induced vascular smooth muscle cell apoptosis is not fully understood, but Ishigami et al noted that apoE inhibition of vascular smooth muscle cell proliferation is mediated through activation of iNOS<sup>11</sup>. However, most of

the molecular mechanisms have not yet been conclusively identified.

Human apoE consists of a single polypeptide chain with 299 amino acids and is best known for its role in the transport of cholesterol and other lipids between peripheral tissue and the liver. However, more direct effects of apoE on the vascular wall may well contribute to arterial protection from atherosclerosis. Many reports focus on the its multi-potentiality. ApoE directs cholesterol efflux mechanisms with the aid of apolipoprotein A-I (apoA-I) and the ATP binding cassette transporter 1. ApoE also has preventive effects on platelet aggregation by facilitating the production of endogenous NO. Platelet aggregation to the internal wall (intima) of normal arteries and capillaries, has pathological consequences by initiating

an inflammatory response in the vessel wall, predisposing the vessel to vascular complications, that include thrombosis, heart disease, myocardial infarction, and stroke<sup>31</sup>. ApoE regulates chronic inflammatory responses and could be protective via direct suppression on the proliferation of T-lymphocytes by internalization of the interleukin 2 (IL-2) receptor<sup>13</sup>. The ability of apoE to inhibit proliferation of endothelial cells and smooth muscle cells by out competing growth factors for interaction with cell surface heparan sulfate proteoglycans further solidifies the role for apoE as being an anti-angiogenic factor in atherosclerosis<sup>33</sup>. The characterization of apoE and its many functions has provided insight into the ultimate potential of this protein as a possible molecular factor in cerebral aneurysm formation.

Caird et al noted that apoA expression in intracranial aneurysms may occur independently of atherosclerosis and its expression in feeding vessels suggests a possible role for apoA in early events leading to aneurysm formation<sup>9</sup>. Recently Yamada et al noted that a genome-wide scan in 29 Japanese families with a high degree of familial clustering revealed 1 suggestive linkage region on chromosome 17cen (inducible nitric oxide synthase gene) and 2 potentially interesting regions on chromosomes 19q13 (the epsilon genotypes of the apoE gene) and Xp22 (the angiotensin I converting enzyme 2 gene)<sup>35</sup>. This suggests possibility that apoE will be one of the potential molecular factors for cerebral aneurysm formation genetically.

In the present study we used an antibody against apoE and

marked positive immunoreactivity is found in the medial smooth muscle cell layer of early aneurysmal change but not detected in the advanced aneurysmal change. These findings may be interpreted that apoE has a possible role in early events of smooth muscle cell layer leading to aneurysm formation. After disappearance of smooth muscle cell layer in advanced aneurysmal change, the role of apoE has finished and expression was not detected in advanced aneurysmal change. But we don't know exact role of apoE in early aneurysmal change. It may promote or prevent the cerebral aneurysm development. Further studies should be aimed at specific hypotheses regarding the potential biological role of this molecule in the pathogenesis of aneurysm.

## Conclusion

ApoE expression in smooth muscle cell layer of the early aneurysmal wall in experimentally induced cerebral aneurysm of rats suggests a possible role for apoE in early events leading to aneurysm formation; these findings require further investigation.

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## Commentary

The authors demonstrated apolipoprotein(apoE) expression in the experimental intracranial aneurysm. They observed in 1 month group after receiving a cerebral aneurysm induction procedure that only 3 of 5 rats showing early aneurysmal change revealed immunoreaction indicating apoE,

and suggested that this expression may occur in early events leading to aneurysm formation.

As authors mentioned, an association between apoA and apoE expression and early aneurysmal change has been already demonstrated in human studies. The authors tried to find their association in the rats using histological and immunohistochemical analysis and supported the previous results. Owing to small numbers, it is not possible to attach much importance to the results for apoE expression in the cerebral aneurysm. Also, apoE plays an important role in the response of the CNS to acute injury, possibly involving scavenging of cholesterol and lipids from injured tissue<sup>1,2)</sup>. The extrapolation that apoE expression in an experimental aneurysm induced by mechanical manipulation is associated with early aneurysm formation may not be justified because acute arterial injury itself by such hemodynamic stress can influence apoE expression in the smooth muscle cell layer.

The pathogenesis of aneurysm are complex, and also the mechanisms by which atherosclerosis to aneurysm formation have not been fully elucidated. Additionally, apoE is involved in several different biologic processes. Further and larger studies are clearly warranted to provide evidence or mechanism for their causal relationship.

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