The efficacy of apolipoprotein E deficiency in cerebral aneurysm formation

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Abstract. Subarachnoid hemorrhage due to the rupture of a cerebral aneurysm is a life-threatening disease. Despite this, the detailed mechanisms underlying the initiation and progression of cerebral aneurysm are unclear. The relation of hypercholesterolemia and apolipoprotein E (ApoE) to cerebral aneurysm formation, has been unclear until now. We used, in the present study, a previously established cerebral aneurysm model of rats and mice whose histological features were closely similar to human cerebral aneurysms. ApoE protein was expressed mainly in the endothelial cells of arterial walls both in control arteries and cerebral aneurysms. The expression of ApoE was reduced during aneurysm formation in the immunohistochemistry. The mRNA expression of ApoE in arterial walls was not different between the controls and cerebral aneurysms. Owing to the deficiency of ApoE, mice presented marked hypercholesterolemia, but there was no difference in cerebral aneurysm formation. In the present study, we clarified that ApoE was not responsible for cerebral aneurysm formation.

Introduction

Subarachnoid hemorrhage is one of the most severe forms of stroke and is a life-threatening disease, mainly due to the rupture of cerebral aneurysms. The mortality and morbidity associated with subarachnoid hemorrhage are quite high despite modern technological advancement and intentional treatment. Thus, prevention of the rupture of cerebral aneurysms is important for public health. Despite the seriousness of cerebral aneurysms, the detailed mechanisms underlying their initiation, progression and rupture are not fully understood. We previously established experimentally induced cerebral aneurysm models of rats (1) and mice (2). Using these useful models, we clarified some of the mechanisms underlying the progression of cerebral aneurysms (3-6). In the field of abdominal aortic aneurysm (AAA), aneurysms are shown to initiate and progress from arteriosclerotic changes in arterial walls. However, in pathological studies of cerebral aneurysms, there appear to be no apparent arteriosclerotic changes in aneurysm walls. There is no direct experimental evidence showing a role for arteriosclerosis or hypercholesterolemia in cerebral aneurysm formation. Apolipoprotein E (ApoE) is one of several lipoprotein transfer proteins. The main function of ApoE is receptor-mediated lipoprotein removal from blood. ApoE knockout mice (7) are a well-established animal model of arteriosclerosis and hypercholesterolemia (8). Widely used AAA models of mice are induced in ApoE knock out mice (9-11) because arteriosclerotic changes in arterial walls are a critical pathological feature of AAA.

ApoE protein was found to be expressed in reactive astrocytes, degenerating neurons, and macrophages after cerebral ischemia (12,13), and several studies have supported a protective role of endogenous ApoE against ischemic injury (14-17). ApoE deficiency in mice is known to show a protective effect on ischemic injury. Kitagawa *et al* (18) showed that oxidative stress generated by ischemia-reperfusion was ameliorated in ApoE knockout mice.

In this study, we analyzed the expression of ApoE in experimental aneurysm walls in rats, and the efficacy of ApoE deficiency in experimental cerebral aneurysm formation in mice. We report the role of hypercholesterolemia and ApoE in cerebral aneurysm formation.

Materials and methods

Human samples. Human cerebral aneurysm samples were obtained from 4 patients who underwent neck clipping for unruptured aneurysms with informed consent. As a control,

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we used the middle cerebral artery (MCA) (n=2) obtained at STA-MCA bypass surgery. Paraffin sections (4 μm) were cut and mounted on slides. After deparaffinization and blocking of endogenous peroxidase activity with 0.3% H_2O_2, primary antibody for ApoE [mouse polyclonal anti-ApoE antibody (Santa Cruz Biotechnology, Santa Cruz, CA)] were incubated for 30 min at room temperature followed by incubation with biotin-labeled secondary antibody for 30 min at room temperature. Slides were then incubated with streptavidin-conjugated peroxidase. Finally, the signal was detected using the 3,3'-diaminobenzidine system (Dako, Carpinteria, CA). Nuclear staining was performed by hematoxylin solution. As a negative control, we performed immunohistochemistry without a primary antibody.

Induction of experimentally induced cerebral aneurysms in rats. Rat cerebral aneurysms were induced as previously described by Nagata *et al* (1). After the induction of pentobarbital anesthesia (50 mg/kg i.p.), the left common carotid artery and posterior branches of the bilateral renal arteries were ligated at the same time with 10-0 nylon in 7-week-old male Sprague-Dawley rats. Animals were fed special food containing 8% sodium chloride and 0.12% ß-aminopropionitrile (BAPN; Tokyo Chemical, Tokyo, Japan), an inhibitor of lysyl oxidase that catalyzes the cross-linking of collagen and elastin.

Animal care and experiments complied with the Japanese community standards on the care and use of laboratory animals.

Immunohistochemistry. One month (n=11) or three months (n=10) after aneurysm induction, all rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. As a control, age-matched male Sprague-Dawley rats were sacrificed as described above. The anterior cerebral artery/olfactory artery (ACA/OA) bifurcation was stripped, embedded and frozen. Sections (5 μm) were cut and mounted on silane-coated slides. After blocking with 5% donkey serum, the slides were incubated with primary antibodies [goat polyclonal anti-ApoE antibody (Santa Cruz), mouse monoclonal anti-smooth muscle α actin antibody (Lab Vision, Fermont, CA), mouse polyclonal anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1) (Santa Cruz), and rabbit polyclonal anti-CD68 antibody (Santa Cruz)] for 1 h at room temperature followed by incubation with fluorescence-labeled secondary antibodies [FITC-conjugated donkey anti-goat IgG antibody, Cy3-conjugated donkey anti-mouse IgG antibody, Cy3-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, Baltimore, MD)] for 1 h at room temperature. Then, the slides were covered with Permafluor (Immunotec, Marseille, France) and excited for fluorescence by illumination through a fluorescence microscope system (BX51N-34-FL-1, Olympus, Tokyo, Japan).

RNA isolation, reverse transcription and PCR of rats. Two weeks, one month or three months after aneurysm induction, rats were sacrificed as described above. Total RNA from the whole Willis ring was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). Extraction was performed according to the manufacturer's instructions. Using Sensiscript reverse transcriptase (Qiagen), total RNA was converted into cDNA, which was then used in each PCR reaction. PCR was performed using HotStart Taq polymerase (Qiagen). α-actin was used as an internal control. The primer sets used were: 5'-gaccctggaggctaaggact-3' and 5'-ccc acagagccttcatcttc-3' for ApoE, and 5'-aagtccctcaccctcccaa aag-3' and 5'-aagcaatgctgtcaccttccc-3' for α-actin. The conditions for PCR were: 40 cycles of 95˚C for 30 sec, 50˚C for 30 sec and 72˚C for 30 sec. PCR products were separated by electrophoresis in 2% agarose gels. Two samples in each group were subjected to RT-PCR analysis and three independent analyses were performed. Densitometric analysis included data from 6 samples per group.

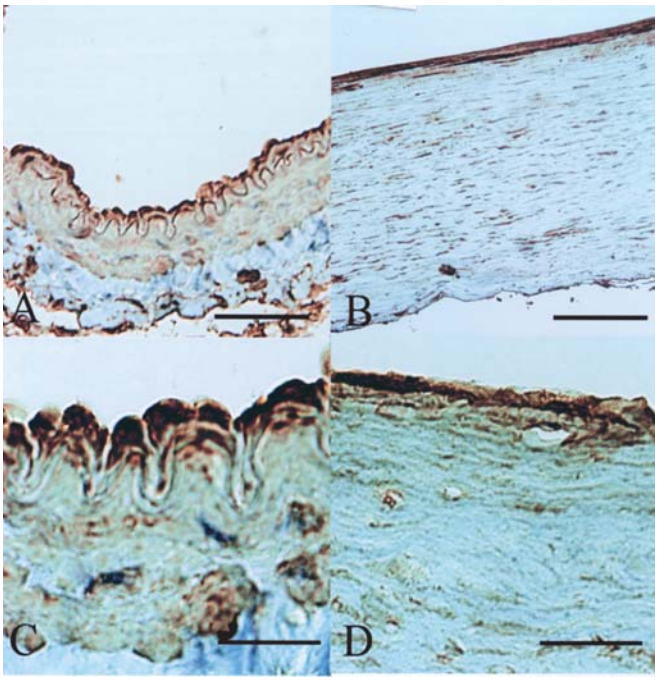

Figure 1. Apolipoprotein E (ApoE) expression in a human aneurysm wall. (A, C) Control middle cerebral artery and (B, D) the human aneurysm wall. Bar, 200 μm (A, B), 50 μm (C, D). Immunohistochemical studies indicated that ApoE-immunoreactive cells were predominantly present in the endothelial cell layer and faintly detected in smooth muscle cells of the media of cerebral arterial walls (A, C). ApoE immunoreactivity was also detected in the endothelial layer and scattered ApoE immunoreactive cells in the media were shown in human cerebral aneurysms (B, D).

Induction of experimentally induced cerebral aneurysms in mice. ApoE$^{-/-}$ mice were purchased from Taconic Farms Inc. (Germantown, NY). At 7 weeks of age, cerebral aneurysms were induced as previously described by Morimoto *et al* (2). Briefly, the left common carotid artery was ligated under 2% fluothane anesthesia. After one week, the posterior branches of the bilateral renal artery were ligated. Animals were fed the same food as used in the rat cerebral aneurysm model. After five months of aneurysm induction, mice were sacrificed and frozen sections were made as described above. After Elastica van Gieson staining, aneurysm formation at the ACA/OA bifurcation was assessed under a light microscope. Aneurysm refers to an outward bulging of the arterial wall

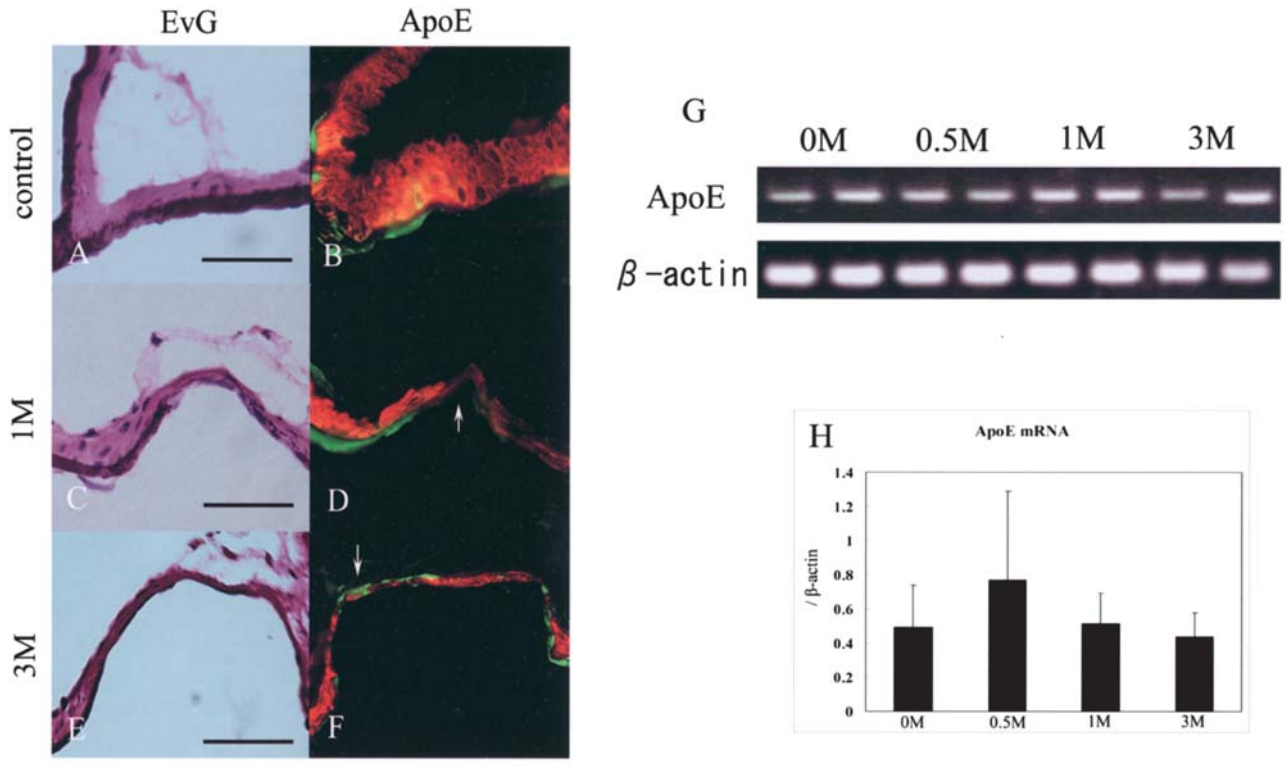

Figure 2. Histological changes and apolipoprotein E (ApoE) expression in experimental aneurysm walls. (A, B) Experimental aneurysm wall of the control rat. Bar, 50 μm. Elastica van Gieson staining (EvG) of normal anterior cerebral artery/olfactory artery (ACA/OA) bifurcation (A). ApoE immunoreactivity was observed in the endothelial layer of the ACA/OA bifurcation (B). (C, D) The aneurysm wall of rats one month after aneurysm induction. Bar, 50 μm. EvG staining indicates early aneurysmal changes in the ACA/OA bifurcation (C). ApoE immunoreactivity was shown in the endothelial layer of the ACA/OA bifurcation, but was lacking in the area of aneurysmal change (D, arrow). (E, F) The aneurysm wall of rats three months after aneurysm induction. Bar, 50 μm. EvG staining indicates developed aneurysmal changes in the ACA/OA bifurcation (E). ApoE immunoreactive cells were also located in the endothelial layer of the ACA/OA bifurcation, but were lacking in the area of aneurysmal change (F). In addition, ApoE expression was detected in the adventitia of aneurysmal walls (F, arrow). Cy3 shows the expression of smooth muscle α actin (red) and FITC shows the expression of ApoE (green). (G) Representative results of the PCR study. ß-actin was used as the internal control; 0 M, control rats; 0.5 M, two weeks after aneurysm induction; 1 M, one month after aneurysm induction; and 3 M, three months after aneurysm induction. (H) Densitometric analysis of ApoE mRNA expression (n=6 in each group). Data (means ± SD) were analyzed by one-way ANOVA followed by the Fisher's test. The expression of ApoE mRNA did not significantly differ among the 4 groups.

detected by light microscopy. Early aneurysmal change refers to a lesion with discontinuity of the internal elastic lamina, without apparent outward bulging of the arterial wall. Advanced aneurysm refers to an obvious outward bulging of the arterial wall with the fragmentation or disappearance of the internal elastic lamina. Three independent researchers assessed the histopathological changes.

The concentration of total cholesterol in mice serum. Mice serum was collected from the right atrium when mice were sacrificed. After centrifugation, serum was frozen at -80˚C until measurement. The concentration of total cholesterol in serum was measured using the Cholesterol E-test (Wako Pure Chemical Industries Ltd., Tokyo, Japan) according to the manufacturer's directions.

Statistical analysis. Values were expressed as the means ± SD. Statistical analysis was performed using the Student's t-test for a two-group comparison and one-way ANOVA followed by the Fisher's test for a multiple comparison. The incidence of aneurysmal changes was analyzed by the use of the Fisher's exact test. Differences were considered statistically significant at $P<0.05$.

Results

ApoE expression in human aneurysm walls. Immunohistochemical studies indicated that ApoE immunoreactive cells were predominantly present in the endothelial cell layer and faintly detected in smooth muscle cells in the media of middle cerebral arterial walls (Fig. 1A and C). In cerebral aneurysms, the internal elastic lamina was thin or absent. The thickness of the media was reduced and that of the endothelial layer was increased. ApoE immunoreactivity was detected in the endothelial layer, and scattered ApoE immunoreactive cells in the media were shown in human cerebral aneurysms (Fig. 1B and D). The intensity of endothelial ApoE immunoreactivity was weaker in aneurysms than in the control samples.

ApoE expression in the aneurysm walls of rats. In the control rats, ApoE immunoreactive cells were predominantly present in the endothelial cell layer and faintly detected in smooth muscle cells of the media of cerebral arterial walls (Fig. 2A and B). One month after aneurysm induction, early aneurysmal changes, including smooth muscle thinning and fragmentation of the internal elastic lamina, were observed at the anterior

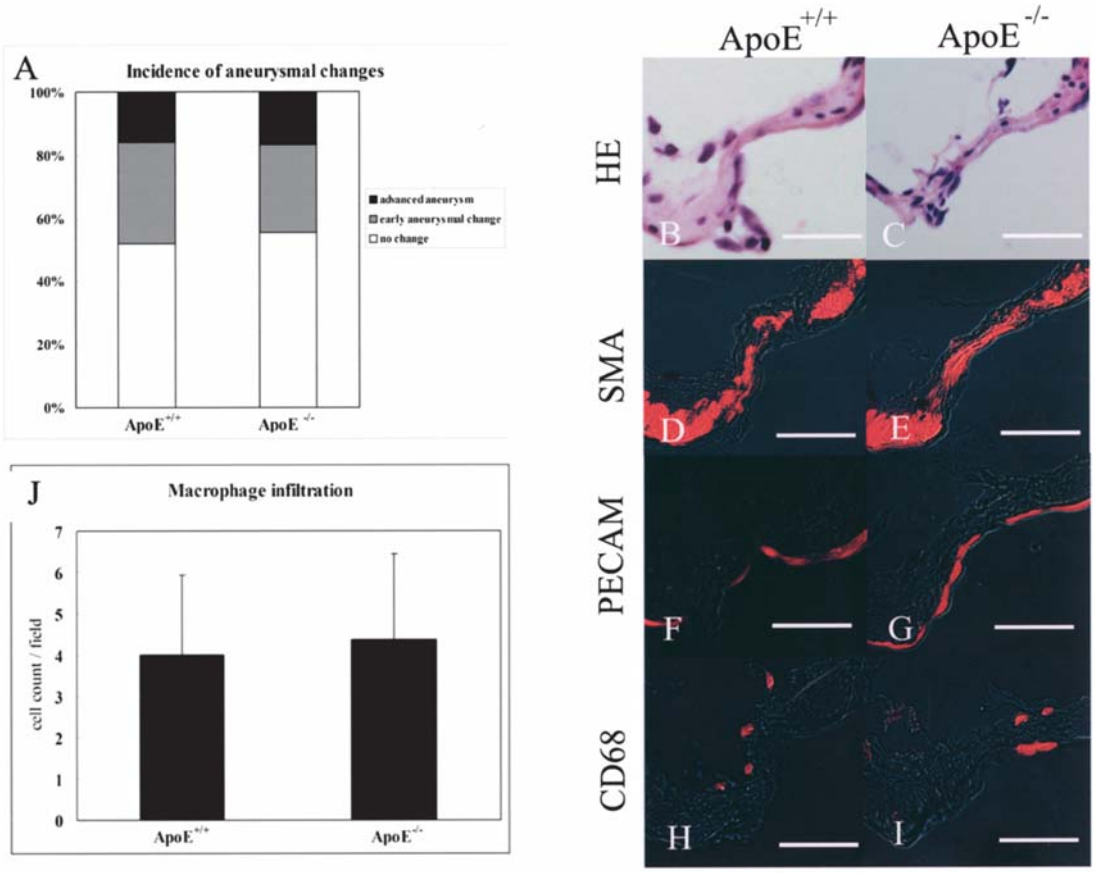

Figure 3. Results of cerebral aneurysm formation and pathohistological examination in apolipoprotein E (ApoE)-deficient mice. (A) The result of cerebral aneurysm formation. Of the 25 $ApoE^{+/+}$ mice, a total of 12 (48%) developed aneurysmal changes and 4 (16%) developed advanced aneurysms. Of the 18 $ApoE^{-/-}$ mice, a total of 8 (45%) developed aneurysmal changes and 3 (17%) developed advanced aneurysms. There was no significant difference in cerebral aneurysm formation between the 2 groups. (B, C) Hematoxylin and eosin (HE) staining of advanced experimental aneurysms in $ApoE^{-/-}$ and $ApoE^{+/+}$ mice. Bar, 50 μm. No histological differences were detected. (D, E) Smooth muscle α actin expression in the aneurysm walls of $ApoE^{+/+}$ (D) and $ApoE^{-/-}$ mice (E). Bar, 50 μm. The expression and distribution of actin were similar between the two lines. (F, G) PECAM expression in the aneurysm wall of $ApoE^{+/+}$ (F) and $ApoE^{-/-}$ mice (G). Bar, 50 μm. The expression and distribution of PECAM were similar between the two lines. (H, I) CD68 expression in the aneurysm walls of $ApoE^{+/+}$ (H) and $ApoE^{-/-}$ mice (I). Bar, 50 μm. The expression and distribution of CD68 were similar between the two lines. Cy3 shows the expression of smooth muscle α actin, PECAM and CD68. (J) The number of macrophages infiltrating the aneurysm wall per 100 μm^2 in sections. Macrophage was the main inflammatory cell in aneurysm walls. There was no difference in macrophage infiltration between $ApoE^{+/+}$ (4.0±1.9 cells/100 μm^2) and $ApoE^{-/-}$ mice (4.4±2.1 cells/100 μm^2).

cerebral artery/olfactory artery (ACA/OA) bifurcation by Elastica van Gieson staining (EvG) (Fig. 2C). In these specimens, ApoE immunoreactivity was indicated in the endothelial layer and was faintly detected in the adventitia (Fig. 2D). The ApoE immunoreactivity in the endothelial layer of arterial bifurcations displaying aneurysmal change was weak. Three months after aneurysm induction, histological changes became more obvious than at one month after aneurysm induction (Fig. 2E). In the area of aneurysmal formation, endothelial ApoE immunoreactivity was fainter than that in any other region. In the adventitia of arterial bifurcations displaying aneurysmal change, strongly ApoE-immunoreactive cells were observed (Fig. 2F, arrow).

RT-PCR for ApoE. Next we assessed the expression of ApoE mRNA in cerebral arteries during experimental aneurysmal formation. The results of RT-PCR analysis indicated that the total amount of ApoE mRNA expression was not significantly different among the control, 0.5, 1 and 3 months after aneurysm induction (Fig. 2G and H).

Cerebral aneurysm formation in ApoE-deficient mice. Cerebral aneurysms were induced in control C57/B6 and ApoE-deficient mice. The anatomical structure of the circle of Willis in both groups, assessed by light microscopic analysis (n=10 in each group), was not different (data not shown). Of the 25 C57/B6 mice, 4 developed advanced aneurysms (16%) and 8 (32%) developed early aneurysmal changes. Of the 18 ApoE-deficient mice, 3 (17%) developed advanced aneurysms and 5 (28%) developed early aneurysmal changes. The incidence of cerebral aneurysms was not different between the groups (Fig. 3A). Furthermore, the incidence of advanced aneurysm was also not different between the two groups.

Histopathological analysis of experimental aneurysm in ApoE-deficient mice. We compared the histopathological features of experimental aneurysms between the control and ApoE-deficient mice. Hematoxylin and eosin staining indicated that the histological features of experimental aneurysm were not different between the two groups (Fig. 3B

Table I. Biochemical data from $ApoE^{+/+}$ and $ApoE^{-/-}$ mice five months after aneurysm induction.

	$ApoE^{+/+}$	$ApoE^{-/-}$	P-value
Total protein (g/dl)	5.8±0.2	6.4±0.4	n.s
Albumin (g/dl)	3.5±0.11	3.3±0.11	n.s
BUN (mg/dl)	21.6±4.1	26.8±0.2	n.s
Cre (mg/dl)	0.11±0.012	0.1±0.035	n.s
AST (IU/l)	135±73	161±81	n.s
ALT (IU/l)	25.3±7.0	34.7±7.6	n.s
ALP (IU/l)	3.5±0.11	3.5±0.11	n.s
LDH (IU/l)	422±231	767±218	n.s
AMY (IU/l)	2812±191	2240±237	0.0310
Na (mEq/1)	155±1.2	151±2.3	n.s
K (mEq/1)	6.1±0.50	7.2±0.53	n.s
Cl (mEq/1)	106±2.0	102±3.5	n.s
Ca (mg/dl)	<10	<10	n.s
P (mg/dl)	7.7±1.0	9.4±0.53	n.s
T-Bil (mg/dl)	0.062±0.011	0.073±0.023	n.s
Glu (mg/dl)	256±54.1	299±31.1	n.s
T-Cho (mg/dl)	113±9.2	619±64	<0.0010
TG (mg/dl)	50.7±23.0	73.7±26.0	n.s
LDL-Cho (mg/dl)	10.7±1.16	109±11.0	<0.0010
HDL-Cho (mg/dl)	62.7±9.0	18.7±3.11	0.0013

Total serum cholesterol (P<0.001) and LDL-cholesterol (P<0.001) were significantly higher in the $ApoE^{-/-}$ mice than in the $ApoE^{+/+}$ mice. HDL-cholesterol (P=0.0013) and amylase (P=0.031) were also significantly higher in the $ApoE^{-/-}$ mice than in the $ApoE^{+/+}$ mice. n.s, statistically not significant (P>0.05).

and C). In addition, we analyzed the expression of smooth muscle α actin (SMA), platelet-endothelial cell adhesion molecule (PECAM) and CD68, which are smooth muscle cell, endothelial cell and macrophage markers, respectively. The distributions of SMA, PECAM and CD68 were not different between the two groups (Fig. 3D-I). Furthermore, we analyzed the number of CD68-positive cells in aneurysm walls. The difference between the groups was not significantly different (Fig. 3J).

Biochemical data in control and ApoE-deficient mice. We measured biochemical data in the control and ApoE-deficient mice. All data are summarized in Table I. In ApoE-deficient mice, total serum cholesterol (632±153 mg/dl, n=3) was significantly (P<0.01) higher than that in the control mice (66.8±17.8 mg/dl, n=3).

Discussion

In our study, we reported on the expression and distribution of ApoE in cerebral arteries in human cerebral aneurysms, and during experimental cerebral aneurysm formation in rats. In addition, we showed the effect of ApoE deficiency in mice against cerebral aneurysm formation. ApoE immunoreactivity was mainly observed in the endothelial layer, both in human and control rat arteries. During aneurysm formation, endothelial immunoreactivity for ApoE was reduced. ApoE deficiency did not affect experimental cerebral aneurysm formation.

Subarachnoid hemorrhage is one of the most severe forms of stroke and is a life-threatening disease, mainly due to the rupture of cerebral aneurysms. The mortality and morbidity associated with subarachnoid hemorrhage is quite high despite modern advanced technology, intentional treatment and considerable effort. Thus, cerebral aneurysms represent a grave disease. However, despite its seriousness, the detailed mechanisms underlying the initiation, progression and rupture of aneurysms are not fully understood.

We established experimentally induced cerebral aneurysm models of rats and mice in 1978 (19) and 2002 (2). These models are very useful because cerebral aneurysms are spontaneously induced without direct handling of cerebral arteries. Moreover, aneurysms were induced at arterial bifurcation sites by hemodynamic stress similar to human aneurysms (4), and the pathological findings in these models, such as the degeneration of the internal elastic lamina and the thinning of media, were shown to be similar to those in human aneurysms (20). Using this useful model, we clarified some of the mechanisms underlying the progression of cerebral aneurysms (3,5,6).

In the field of abdominal aortic aneurysm (AAA), aneurysms have a strong relation with arteriosclerotic changes and vascular inflammation of arterial walls. ApoE is one of several lipoprotein transfer proteins. The main function of ApoE is receptor-mediated lipoprotein removal from blood. ApoE knockout mice (7) are a well-established animal model of arteriosclerosis and hypercholesterolemia (8). Widely used AAA models are induced in ApoE knock out mice (9-11) because arteriosclerotic changes in the arterial walls are a critical pathological feature of AAA. However, from histopathological studies of cerebral aneurysms, there has been no apparent evidence of arteriosclerotic changes in aneurysm walls. Moreover, cerebral aneurysms usually form at the arterial bifurcation in both human and experimental animals. This is the critical difference between cerebral aneurysms and AAAs, which form at the side wall of the aorta, not at bifurcation sites. Thus, some different mechanisms might be involved in cerebral aneurysm formation compared with AAA formation. Hypercholesterolemia is not a risk factor of cerebral aneurysms (21-24) unlike AAA. However, direct experimental results for this fact are absent.

First, ApoE is expressed consistently in arterial walls and aneurysm walls both at the protein level (Fig. 2A-F) and at the mRNA level (Fig. 2G and H). The ApoE-deficient mice in this study showed marked hypercholesterolemia (Table I). ApoE-deficient mice developed cerebral aneurysmal changes after aneurysm induction, but there was no difference between them and the C57/B6 mice (Fig. 3A). From these results, cerebral aneurysm formation had no relation with hypercholesterolemia and ApoE expression. Regarding ApoE, analysis of the polymorphisms present in cerebral aneurysms has been performed previously. However, the authors of these studies failed to show a positive relationship with cerebral

aneurysms (25,26). Our data supported their results. The relation between ApoE genotype and outcome after subarachnoid hemorrhage was also reported (27). In our mouse model, we did not study the rupture rate due to the lack of appropriate methods for evaluating subarachnoid hemorrhage such as CT scan and outcome after subarachnoid hemorrhage.

In addition to hypercholesterolemia, ApoE plays a role in the central nervous system. Aoki *et al* (12) reported that neurons and degenerated axons in and around the ischemic foci contained ApoE immunoreactivity, in addition to astrocytes and microglia. A growing body of evidence suggests a role for endogenous ApoE in modifying the central nervous system's response to acute injury (28). In preclinical models of focal and global ischemia, the absence of endogenous ApoE has been associated with a worsening of both histological and functional outcomes (14,16,17). There are likely multiple mechanisms by which endogenous ApoE plays a protective role in the injured central nervous system, as suggested by prior *in vitro* data demonstrating its isoform-specific antioxidant (27) and neurotrophic effects (29). In addition, ApoE polymorphisms were reported to be independent risk factors for ischemic stroke. In our study, cerebral endothelial immunoreactivity of ApoE was reduced during cerebral aneurysm formation. However, we did not detect a difference in cerebral aneurysm formation between ApoE-deficient mice and wild-type mice. This result indicates that endothelial endogenous ApoE does not play a role in cerebral aneurysm formation. By immunohistochemical analysis, macrophage infiltration did not differ between the two groups. Previously, we showed the importance of macrophage infiltration in aneurysm formation (3). Thus, ApoE deficiency may not have affected aneurysm formation in our model.

In summary, we conducted immunohistochemical studies of ApoE in human and experimental cerebral aneurysms. In addition, we showed that ApoE was not responsible for cerebral aneurysm formation using a previously established model of cerebral aneurysms. In the field of abdominal aortic aneurysm, the detailed mechanisms underlying aneurysm formation have been revealed. However, in the field of cerebral aneurysms, studies of the mechanisms underlying cerebral aneurysm formation have been limited. There is a substantial dissimilarity between these two diseases, and this study provides clues towards the elucidation of the mechanisms underlying cerebral aneurysm formation.

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