A Simple Method of Plaque Rupture Induction in Apolipoprotein E–Deficient Mice

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Objective—The development of a murine model of atherosclerotic plaque rupture.

Methods and Results—The left common carotid arteries of male apolipoprotein E (apoE)–deficient mice (9 weeks old) were ligated just proximal to their bifurcations. After 4 weeks on a standard diet, the mice received polyethylene cuff placement just proximal to the ligated site, and the animals were then processed for morphological studies at specific time points. Ligation of the carotid artery in apoE-deficient mice for 4 weeks induced marked intimal hyperplasia, which is a lipid- and collagen-rich lesion that contains a number of macrophages, T lymphocytes, and smooth muscle cells. Subsequently, the cuff placement evoked intraplaque hemorrhage and plaque rupture with fibrin(ogen)-positive luminal thrombus in this region accompanying a decrease in collagen content as well as an increase in apoptotic cells in the intima within a few days after cuff placement.

Conclusions—We demonstrated the murine model of human plaque rupture, which is simple, fast, and highly efficient. This model would help us not only to understand the mechanism of human plaque rupture but also to assess various already-known and as-yet-unknown agents in the future. (Arterioscler Thromb Vasc Biol. 2006;26:1304-1309.)

Key Words: animal model ■ plaque rupture ■ thrombus ■ apoptosis ■ collagen

It is widely believed that rupture of a vulnerable atherosclerotic plaque leads to acute coronary events and stroke. The vulnerable plaque is generally composed of an atrophic fibrous cap, a lipid-rich necrotic core, the accumulation of inflammatory cells,1,2 and imbalance between extracellular matrix synthesis and degradation resulting in decreased extracellular matrix protein content and increased proteinases, including matrix metalloproteinases (MMPs).3–5 However, the exact mechanisms involved in the plaque rupture remain unknown.

Pathological animal models are valuable in the research on human diseases and their therapy. Mice are appropriate laboratory animals because of their small size, which makes them easy to manage and feed, and their superior potential for reproduction. Furthermore, recent methods of analysis and alterations of genes have enlarged their potential as model animals.

The apolipoprotein E (apoE)–deficient mouse has become established as a model of hypercholesterolemia and atherosclerotic lesion development.6,7 Although several plaque rupture models using the apoE-deficient mouse have been proposed, in most of the models, plaque rupture has been seen less frequently even, in old mice after prolonged feeding with very high-cholesterol diets.8,9 More recently, Johnson et al reported that after 8 weeks of fat feeding of apoE-deficient mice, 62% of animals exhibited acute plaque rupture, which is defined as a visible breach in the cap with intraplaque hemorrhage, in the brachiocephalic artery.10 In addition, in most of the models, there is no convincing evidence of the formation of platelet and fibrin-rich occlusive thrombus at the site of presumed rupture,8–10 which is characteristic of the human plaque rupture leading to coronary heart disease and stroke.

In the present study, we propose a novel murine model of atherosclerotic plaque rupture associated with not only intraplaque hemorrhage but also luminal thrombus.

Materials and Methods

Mice

The male apoE-deficient mice (Jackson Laboratory, Bar Harbor, Me) used in this study were 8 weeks old and weighed between 21 and 25 g. Mice were provided with a standard diet (Oriental Yeast) and tap water ad libitum throughout the experimental period. All animal experiments were performed in accordance with the guidelines on animal care of Nagoya University Graduate School of Medicine.

Animal Surgery

The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Dainippon Pharmaceutical). The schematic diagram of the operation is presented in Figure 1. Ligation...
of the common carotid artery in 9-week-old mice was performed just proximal to their bifurcations according to a procedure described previously.11 Four weeks after ligation, a polyethylene cuff (length 2 mm; inside and outside diameter 0.580 mm and 0.965 mm, respectively; Becton Dickinson) was applied just proximal to the ligated site according to the method described previously.12 In the control group, the dissection of the left common carotid artery from the surrounding connective tissue was performed without cuff placement.

**Tissue Collection and Processing**

Just before (0 day) and 2, 4, or 7 days after the cuff placement, mice were anesthetized by intraperitoneal pentobarbital injection. Mice were perfused through the left cardiac ventricle with isotonic saline and 4% paraformaldehyde in 0.01 mol/L phosphate buffer, pH 7.4, under physiological pressure. The carotid arteries were removed and immersed in fixative for 16 hours (4°C). After the removal of the cuff, each vessel was embedded in optimal cutting temperature compound (Sakura Finetechanical), snap-frozen in liquid nitrogen, and stored at −20°C until use.

**Histological and Immunohistochemical Staining**

Cross cryosections (6 μm) were prepared from the carotid artery and stained routinely with hematoxylin and eosin (H&E), oil red O for lipid, and picrosirius red and Masson’s trichrome for collagen. The corresponding sections on separate slides were used for the immunohistochemical staining. The sections were preincubated with 5% serum and then incubated with antibodies against smooth muscle cell actin (αSMC actin; 1:50; Sigma-Aldrich), fibrin(ogen) (1:200; DAKO), single-stranded DNA (ss-DNA; 1:200; DAKO), cleaved caspase-3 (1:100; Cell Signaling Technology), macrophages (1:40; BD Pharmingen), T cells (1:200; Sigma-Aldrich), CD 45 (1:100; BD Pharmingen), neutrophils (1:50; Serotec), and MMP-2 (MMP-2; 1:200; Fuji Chemical Co) and MMP-9 (1:100; Chemicon). Immunohistochemical staining was visualized using an ABC kit (Vector Laboratories) according to manufacturer instructions. Levamisole (Vector Laboratories) was used as the inhibitor of endogenous alkaline phosphatase. The counterstaining for the nucleus was performed with Mayer’s hematoxylin. For identification of apoptotic cell types, we combined the double immunostaining with antibody against ss-DNA13 with an antibody against αSMC actin or macrophages using protocols provided by the manufacturers. Nuclear counterstaining was performed with methylgreen.

**Morphometric Analysis**

By means of H&E staining, the plaque rupture at the intracuff region of the carotid artery was identified by cracks in the neointima and intraplaque hemorrhage. The presence of cracks, thrombus, or intraplaque hemorrhage of each vessel was determined by analysis of sections at 60-μm intervals. The apoptotic index was determined by using antibody against ss-DNA in the arterial neointima and expressed as the percentage of apoptotic cells within the total number of cells in a given area. The specimens were examined histologically using picrosirius red staining for collagen. The cross-sectional apoptotic cells and collagen area were quantitatively determined by using Scion Image Analysis software (Scion Co).

**Statistical Analysis**

Data were represented as means±SD. One- or 2-way ANOVA followed by post hoc testing (Scheffe test) was used for statistical analysis where appropriate at P<0.05.

**Results**

**Plaque Rupture in the Carotid Artery**

No animals died during this experiment, and no behavioral changes were noted.

Neointima formation hardly occurred just before the ligation in the carotid artery of an apoE-deficient mouse (Figure 2A). At 4 weeks after ligation, the constant neointima lesion with the residual lumen was induced in the carotid artery (Figure 2B), which was mainly composed of lipid-filled macrophages and T cells (Figure 2C through 2E). αSMC-positive cells were detected in the fibrous cap region of the neointima at 4 weeks after the operation (Figure 2F). No intraplaque hemorrhage or evidence of plaque rupture was detected at this time. At 2 days after the cuff placement at the ligated artery, a marked intraplaque hemorrhage and superficial αSMC-positive cells were observed in the neointima (Figure 2G and 2H). At 4 days after the cuff placement, the cracks of neointima and the thrombus formation in lumen were constantly observed at overall intracuff region, and the immunoreactivity of αSMC actin was attenuated at the crack of the neointima in the intracuff region of the carotid arteries (Figure 2I, 2I inset, and 2J). Fibrin(ogen), leukocytes, and neutrophils were detected in the carotid arteries at 4 days after the cuff placement (Figure 2K through 2M). In some lesions, the occlusive luminal thrombi were also detected. The proportions of intraplaque hemorrhage and cracks of neointima accompanying the thrombus are summarized in the Table. At 7 days after the cuff placement, dramatic disruption of neointima and occultive thrombus formation were detected in intracuff region of the carotid artery in all animals (Figure 2N; n=6). It should be noted that cuff placement for 1 hour followed by removal of cuff did not induce plaque rupture and intraplaque hemorrhage at day 4 after cuff placement (Figure 2O), suggesting that the surgical manipulation of the carotid artery is not involved in the rupture and intraplaque hemorrhage.

**Analysis of Apoptotic Cells**

A few apoptotic cells were detected in the neointima lesion at 4 weeks after ligation (ie, just before cuff placement; Figure 3A), and the cuff placement resulted in a significant increase in apoptotic cells at 2 and 4 days (Figure 3B and 3C). At the cracks of neointima, the accumulation of apoptotic cells in a fibrous cap was observed at day 4 (Figure 3C). Immunoreactivities were not observed in negative control specimen (Figure 3D). Immunostaining for cleaved caspase-3, another index for apoptotic cell, confirmed the results of using antibody against for ss-DNA. Cleaved caspase-3-positive cells were increased after cuff placement (Figure 3E and 3F). The apoptotic index using ss-DNA antibody was calculated by image analysis and is summarized in Figure 3I, and the
index using caspase-3 antibody is as follows: 0 days, n=16, 2.7±1.8%; 2 days, n=17, 11.4±7.7%; 4 days, n=21, 9.9±5.2%. Double immunostaining with specific antibody to ss-DNA/ssMC actin or ss-DNA/macrophages indicated that the apoptosis was induced in SMCs in a fibrous cap and macrophages in the neointima (Figure 3G and 3H).

Collagen content in neointima and immunoreactivity of MMP-2 and MMP-9 accumulation of collagen was observed in neointima at 4 weeks after ligation (Figure 4A and 4D), and the collagen staining was greatly decreased at 2 (Figure 4B and 4E) and 4 days (Figure 4C and 4F) after cuff placement. The area of collagen staining was analyzed by image analysis and is summarized in Figure 4G. Immunoreactivity for MMP-2 was detected in both the neointimal, mainly at the fibrous cap region, and medial regions at 2 days after cuff placement (Figure 4H). Staining for MMP-9 was mainly observed in plaque region of intima at 2 days after cuff placement (Figure 4I).

**Discussion**

In the present study, we provide a novel model for progress of atherosclerotic plaque vulnerability and rupture in apoE-deficient mice, which is simple, fast, and highly efficient. We used the ligation technique to induce neointimal hyperplasia, which is not a spontaneous atherogenesis but, if anything, a kind of remodeling lesion after vascular injury. Ligation of the carotid artery in apoE-deficient mice after 4 weeks on a standard rodent chow without high fat induced marked intimal hyperplasia, which is a lipid- and collagen-rich lesion that contains a number of macrophages, T lymphocytes, and SMCs. Subsequently, the cuff placement evoked intraplaque hemorrhage and plaque rupture in this lesion, accompanying a decrease in collagen content within a few days after cuff placement. Formation of fibrin(ogen)-positive thrombus was detected in the lumen associated with the occurrence of plaque rupture. Furthermore, apoptotic cells were increased after cuff placement, and some of them were identified as macrophages or SMCs. Immunoreactivities of MMP-2 and MMP-9 were observed in the intima. It should be noted that some intraplaque hemorrhages were detected in areas outside
of the cuff placement, especially close to the cuff placement, but no lesions of luminal thrombus or neointima cracks were observed in the regions outside of the cuff placement. Endothelial injury or physical disruption of endothelial lining possibly induced by the cuff placement was not involved in the plaque rupture in this model because cuff placement for 1 hour followed by removal of cuff did not induce plaque rupture.

We believe that the plaque vulnerability and rupture in this model partially overlap with human disease and improve several disadvantages in previous models of plaque rupture.

Several models of plaque rupture using apoE-deficient mice have been published. In the first atherosclerotic plaque rupture model, rupture was provoked by mechanical injury of the atherosclerotic plaque using a forceps. Others reported that plaque rupture in the brachiocephalic artery was induced by a diet supplemented with high fat for long periods (12 to 15 months). Recently, von der Thüsen et al reported an interesting method of atherosclerotic plaque rupture in apoE-deficient mice that uses a combination of a perivascular cuff placement and transfection with the p53 gene, a tumor suppressor that promotes apoptosis. Furthermore, Johnson et al demonstrated the excellent finding that acutely ruptured plaques were observed in the brachiocephalic arteries of 62% of apoE-deficient mice after 8 weeks of fat feeding; subsequently, this proportion had fallen to 30% after 9 weeks. Some of previous observations suggest that spontaneous plaque rupture in apoE-deficient mice was seen only in older

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![Figure 3](image-url)

**Figure 3.** Presence of apoptotic cells after cuff placement. ss-DNA staining for apoptotic cells before cuff placement (A) and at 2 days (B) and 4 days (C) after cuff placement. Red punctate staining is apoptotic nucleus, and counter staining by hematoxylin for nucleus. Negative control (D) in absence of primary antibody. Immunostaining using cleaved caspase-3 antibody for apoptotic cells before cuff placement (E) and at 2 days (F) after cuff placement. Double immunostaining with antibody to ss-DNA/cSMC (G) actin or ss-DNA/macrophages (H). Black staining is apoptotic nucleus, Red staining is cSMC or macrophages. Counter staining by methyl green for nucleus. I, apoptotic index expressed as the percentage of apoptotic cells within the total number of cells in a given area (values are means±SD, n=14, 16, and 15 of 0d, 2d, and 4d group, respectively, *P<0.05). Bars=100 μm. T, thrombus, f, fibrous cap, arrow and arrow heads indicate crack of fibrous cap and double-stained cells, respectively.

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![Figure 4](image-url)

**Figure 4.** Collagen content in neointima and immunoreactivity of MMP-2 and MMP-9. A through C, Picrosirius red staining viewed under polarized light before cuff placement and at 2 days (2d) and 4 days after cuff placement. D through F, Masson’s trichrome staining of samples before, at 2 days, and at 4 days after cuff placement. G, Quantitative analysis of collagen content. Percentage of positive area for picrosirius red staining within intima was determined by computer analysis. Values are means±SD; n=12, 13, and 15 of 0d, 2d, and 4d groups, respectively. H, I, MMP-2 and MMP-9 staining, respectively. Each lower-right inset illustrates a negative control. F, *P<0.05. Bars=100 μm. T indicates thrombus, and arrowheads indicate crack of fibrous cap.
mice after prolonged feeding with a very high-fat diet. In addition, the presence of luminal thrombi occurs only rarely in mice, and when present, thrombi are mostly not organized and nonocclusive.

There are several limitations in this model, particularly concerning atherosclerotic lesions. We used the method of ligation to induce the neointimal hyperplasia, which represents a mechanically induced remodeled lesion rather than a typical asymmetrical atherosclerotic lesion of apoE deficiency. Although SMCs were distributed within the neointima along the lumen, intimal collagen was deposited ubiquitously. In this experiment, intraplaque hemorrhage was observed after the treatment of cuff placement. Pathologic studies in human tissues have demonstrated that intraplaque hemorrhage is associated with an increased density of intimal microvessels. On the other hand, the presence of plaque neovascularization remains controversial in apoE-deficient mice. Plaque neovascularization has been observed at aortic sinus lesion, but Johnson et al did not confirm intimal neovascularization in the brachiocephalic arteries of nearly 700 apoE-deficient mice. Furthermore, it has been suggested that intraplaque hemorrhage occurs by influx of blood elements through the fibrous cap and may be a consequence of the plaque rupture. However, the existence of microvessels in the neointima induced by the ligation were not examined in this experiment. Further experiments will be required to elucidate the pathogenesis of the intraplaque hemorrhage in this model.

We still do not know the exact mechanisms of the plaque rupture in this model. As described above, we observed a reduction of collagen content, the presence of MMPs, and an increase in apoptotic cells in the plaque lesions before the rupture. These observations may indicate that the degradation of collagen is in part responsible for the plaque rupture in this model. The increase in apoptotic SMCs may have contributed to the reduction of collagen synthesis, leading to the imbalance between extracellular matrix synthesis and degradation, which is a solid basis for plaque rupture. Thus, the sequence of the plaque rupture in this model appears to be analogous to the event in humans. In addition, it is suggested that inflammation is involved in the plaque disruption. In this study, inflammatory cells, macrophages, and T lymphocytes were observed in intima before the cuff placement, and moreover, abundant accumulation of neutrophils, which are the first-phagocyte cells in acute inflammatory response, was detected after the cuff placement. In the previous report, neutrophils were identified at the site of plaque rupture, and neutrophil infiltrates were present in culprit lesions in acute coronary syndromes. Therefore, those above inflammatory cells, especially neutrophils, may participate in mechanisms of plaque rupture in this model. In addition, we showed that the cuff placement induced the plaque disruption or occlusive thrombus accompanying inflammatory reaction in the carotid artery but not in sham operation artery. Several inflammatory cells known to release various proteinases were observed in lesion sites of this model. Furthermore, some of these proteinases may be involved in the pathogenesis of endothelial injury, which may facilitate the plaque disruption or thrombus formation. On the other hand, it is unclear what role is played by apoE deficiency on the plaque rupture in this model. Previous reports showed the anti-inflammatory nature of apoE, and it is possible that apoE deficiency may contribute to plaque rupture through the augmentation of the inflammatory reactions in the vascular walls after cuff placement. However, to obtain direct evidence to support this hypothesis, further investigations will be required using this model.

In the present study, we demonstrated an animal model of human plaque rupture. This method is simple, fast, and highly efficient. We hope that this plaque rupture model will help us not only to understand the mechanism of human plaque rupture but also to assess various already-known and as-yet-unknown agents in the future.

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References


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