Increased Balloon-Induced Inflammation, Proliferation, and Neointima Formation in Apolipoprotein E (ApoE) Knockout Mice

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Background and Purpose—The pathophysiology of vascular lesions after balloon angioplasty remains poorly understood. A major limitation of most experimental studies in this regard is that injury was assessed in healthy arteries. Our aim was to study the effects of hypercholesterolemia in a mouse vascular injury model that mimics human balloon angioplasty.

Methods—Carotid balloon distension was performed in wild-type (WT) mice on a normal diet (ND), in apolipoprotein E–deficient (ApoE<sup>−/−</sup>) mice on ND and in ApoE<sup>−/−</sup> mice fed a high cholesterol diet (CD).

Results—Medial cell death (TUNEL) was elevated in all mice at 1 hour and 1 day after angioplasty without differences between the groups. We found enhanced intimal inflammation (%CD45-positive cells) and vascular cell adhesion molecule-1 expression at 7 days (P<0.05; n≥4) as well as increased proliferation rates (BrdU-index) in ApoE<sup>−/−</sup> CD at 7 and 28 days postinjury (P<0.05; n≥5). Four weeks after injury, these events led to enhanced neointima in ApoE<sup>−/−</sup> CD compared with WT ND mice (intima/media, P<0.001; n≥8). The amount of lesion formation paralleled the incremental increase in total plasma cholesterol in WT ND, ApoE<sup>−/−</sup> ND and ApoE<sup>−/−</sup> CD (P<0.01).

Conclusions—Carotid balloon distension injury in ApoE<sup>−/−</sup> mice on CD induced enhanced inflammation and proliferation leading to increased neointima. Further applications of this microballoon catheter in genetically modified mice will provide opportunities to elucidate molecular mechanisms of vascular lesion formation in a model that reflects clinical balloon angioplasty. This know-how may pave the way to catheter-based interventions of human microvessels in the peripheral or cerebral circulation. (Stroke. 2006;37:2625-2632.)

Key Words: cholesterol ■ angioplasty ■ inflammation ■ apolipoprotein E

Restenosis, the result of neointima formation and remodeling, still decreases the long-term clinical success of percutaneous coronary and peripheral interventions. Local therapies such as drug-eluting stents have successfully reduced the rate of balloon- or stent-induced restenosis. However, many of its molecular mechanisms remain poorly understood. Transgenic technologies in mice provide a powerful tool to address this problem. Furthermore, apolipoprotein E knockout (ApoE<sup>−/−</sup>) mice with or without high cholesterol diet offer a model of marked hypercholesterolemia and atheroma reflecting the context of human atherosclerosis. Therefore, the challenge for vascular biologists in recent years was to establish a murine model of human restenosis. However, as balloon catheters amenable to mouse vessels were not available, the previous types of murine vascular injury such as wire injury, fluid instillation and air desiccation, spring or cuff injury at best tried to imitate balloon injury. Applying the concept of “responsiveness to injury”, it is likely that the nature of the injury determines the relative role of the following molecular and cellular events that ultimately lead to restenosis.

Therefore, we have designed a miniature balloon catheter which enabled us to characterize a murine balloon injury model that mimics balloon angioplasty in humans. By comparing the events after carotid injury between ApoE<sup>−/−</sup> mice on high cholesterol diet (CD) or normal diet (ND) and wild-type (WT) mice on ND, we demonstrate that ApoE<sup>−/−</sup> mice exhibit enhanced inflammation and proliferation leading to increased neointima formation.

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Materials and Methods

Animals and Diets
Male ApoE<sup>−/−</sup> (C57BL/6J) mice and their corresponding WT background strain were obtained from Jackson Laboratory (Bar Harbor, ME) and kept on a regular diet. An additional group of ApoE<sup>−/−</sup> mice was fed a CD (D12108 containing 1.25% cholesterol, Research Diets, New Brunswick, NJ) that was started 10 days before intervention and continued until harvesting. All animal experiments were performed in accordance with institutional guidelines and approved by the local animal committee.

Mouse Carotid Balloon Injury
WT and ApoE<sup>−/−</sup> mice (8 weeks old, around 25 g) were used for experiments. Balloon injury of the left proximal common carotid artery was performed by adapting a previously published protocol. Briefly, animals were anesthetized injecting ketamine (75 to 95 mg/kg; Intervet; Zurich, Switzerland) and xylazine (4 to 8 mg/kg; Rompun, Bayer; Leverkusen, Germany) intraperitoneally. After dissecting the left carotid bifurcation, we ligated the external carotid artery distally, placed clamps on the internal and mid-common carotid artery and introduced the balloon catheter through an arteriotomy on the proximal external carotid artery (Figure 1). After

Figure 1. Carotid balloon distension in the mouse. Scheme depicting the surgical approach to balloon dilatation of the left proximal common carotid artery in the mouse (panel A). After exposing the left carotid bifurcation, the distal external carotid artery (EC) is ligated (panel B). The transient interruption of blood flow by clipping the internal (IC) and mid-common carotid artery (CC) allows insertion of the balloon catheter through an incision in the proximal EC (panel C). After dilating the proximal, nondissestected (nonvisible) CC (panel D), the catheter is withdrawn and the clip on the mid-CC put back in place. After ligating the proximal EC and removing the clips, blood flow through CC and IC is reestablished (panel E). The balloon catheter is stiffened by a guide wire (panel F); the balloon length is 5 mm.
removing the clamp on the common carotid artery, the catheter was advanced to the proximal, nondissected common carotid artery where the balloon was distended for 40 seconds. Balloon size was matched to the weight of the animals using a balloon diameter of 0.77 mm for a 24-g mouse, 0.79 mm for a 25-g, and 0.80 mm for a 26-g mouse. The balloon catheter was specifically manufactured (Schneider, Bulach; then Jomed, Beringen, then Schwager Medica, Winterthur, Switzerland) with a balloon-length of 5 mm and a diameter ranging from 0.72 mm at 8 bars to 0.83 mm at 16 bars. The balloon was stiffened by a guide wire (0.2 mm diameter) and expanded using a water-filled inflation device (Monarch 25; Merit Medical).

**Tissue Harvesting and Processing**

Mice were euthanized 1 hour, 1 day, 7 days and 28 days after balloon distension injury. For the 2 latter time points, animals were injected with BrdU (50 mg/kg, Sigma) intraperitoneally 17 hours, 9 hours and 1 hour before harvesting as described. After puncturing the left ventricle and cutting the right atrium, vessels were rinsed with phosphate-buffered saline (PBS) and perfusion-fixed at 100 mm Hg for 8 minutes using 4% paraformaldehyde (Sigma) in PBS. The injured left and the untouched right common carotid artery were excised after dissecting the adventitia. After postfixation with 4% paraformaldehyde for 2 hours and immersion in 30% sucrose overnight, vessels were embedded in optimal cutting temperature compound (Tissue-Tek), frozen, and stored at −80°C. For immuno-histochemical analyses, vessels were rinsed with normal saline, embedded in optimal cutting temperature compound and frozen without fixation. For scanning electron microscopy, vessels were harvested 1 hour after balloon injury and perfusion fixation was carried out as above using glutaraldehyde 2% buffered with 0.1 mol/L cacodylate (Sigma) for 4 minutes. Vessels were opened longitudinally, dehydrated through series of ethanol, critical point-dried, spattered with 15 nm gold and examined in a scanning electron microscope 505 (Philips).

**Morphology**

Three serial cross-sections (5-μm thickness, 300-μm apart) were taken from the mid-portion of the dilated segment for histomorphological analysis (microscope BX51, Olympus). Corresponding sections were obtained from the untouched right common carotid artery as a negative control. Intimal and medial cell numbers were assessed using 4′,6-diamidino-2-phenylindole (DAPI; 5 μmol/L in PBS; Boehringer). Cell death was determined by TdT-mediated dUTP nick end-labeling (TUNEL; Roche). Cellular proliferation was characterized by staining with a biotin-conjugated mouse anti-BrdU antibody (Zymed, San Francisco, CA) and fluorescein-avidin DCS (1:200; Vector). Proliferation indices (BrdU-I) were calculated as percentage of total cell number. Endothelial cells were characterized by staining with a rat antimouse CD31 (557355, 1:2000; BD Pharmingen, Allschwil, Switzerland), vascular smooth-muscle cells by a biotinylated antirabbit smooth-muscle α-actin antibody (PK-...
Inflammatory cells were identified using a biotin-conjugated rat antimouse CD45 antibody (553077, 1:50; BD Pharmingen). Vascular cell adhesion molecule (VCAM)-1 expression was determined using a rat antimouse antibody (MCA1229, 1:200; Serotec). Morphometric analyses of areas were performed on sections with nuclear staining (DAPI) with the addition of polarized light; sections were photomicrographed (Olympus DP50-CU camera), digitized and analyzed (Analysis 5, SoftImaging System).

Statistical Analysis
All data are expressed as mean±SEM. Statistical significance of differences was calculated using ANOVA with post hoc Tukey test or Student unpaired t test. P<0.05 was considered statistically significant.

Results
Carotid Balloon Distension Injury in Mice Reflects Events After Balloon Angioplasty in Humans
Using a custom-made miniaturized balloon catheter, we performed local over-distension of the nondissected proximal portion of the left common carotid artery (Figure 1). Analyses of cross-sections of the dilated carotid arteries in WT mice showed that balloon distension induced an immediate endothelial cell loss after 1 hour, a marked decrease in medial cells at day 1, followed by cellular infiltrations at day 7 leading to neointima formation at day 28 (Figure 2A).

Our next step was to detail the early events after carotid balloon dilatation in WT mice. Microscopical analyses of carotid arteries 1 hour after balloon angioplasty revealed a platelet monolayer (Figure 2B, top), endothelial denudation (Figure 2B, bottom), as well as increased medial and adventitial cell death (Figure 2C).

Increased Neointima in ApoE /−/− Mice Fed a CD
Hypercholesterolemia is a prominent risk factor leading patients to percutaneous catheter interventions. Therefore, we applied our balloon injury model in the following 3 groups: WT mice on a ND, ApoE /−/− mice kept on a ND and ApoE /−/− mice fed a CD.

Analyses of cross-sections 28 days after injury revealed moderate neointima formation in WT mice that was progressively increased in ApoE /−/− on ND and ApoE /−/− on CD (Figures 3A and 4A and B); neointima formation in injured arteries was increased in all 3 groups as compared with untouched vessels (P<0.05). At this time point, endothelial coverage was complete in all 3 groups (Figure 3B). Lumen narrowing was more pronounced in ApoE /−/− on CD, but did not reach statistical significance. Furthermore, there was no difference in perimeters of external elastic laminae between the groups. However, distension injury induced some enlargement remodeling after 28 days as compared with untouched control arteries (please see supplemental Figures I and II, available online at http://stroke.ahajournals.org). Neointima formation in the different groups paralleled total plasma cholesterol levels (Figure 4C); plasma triglycerides remained unchanged (Figure 4D).

As a next step, we quantified total medial (Figure 4E) and intimal cells (Figure 4F) on carotid cross-sections at different time points after balloon injury and compared them to uninjured control arteries. Measurements at 1 hour and 1 day after balloon injury confirmed endothelial denudation and revealed a more pronounced decline in total medial cells in ApoE /−/− on CD than in WT mice on ND (P<0.05). As balloon distension induced mechanical disruption of medial cells, chromatin staining identified an increased number of medial nuclei 1 hour after injury as compared with uninjured vessels. By day 7, total medial and intimal cells increased in parallel without a significant difference between the groups. Twenty-eight days postangioplasty, counts of total intimal cells

Figure 3. Balloon angioplasty induces neointima formation. A, Chromatin staining of cross-sections obtained 28 days postinjury from WT mice on ND (left), ApoE /−/− on ND (middle) and ApoE /−/− on CD (right). Arrows indicate the internal elastic lamina that delineates the neointima as its outer border. DAPI staining plus polarized light; bar=100 μm. B, Anti-CD31 staining identifies an intact endothelial layer in all 3 groups 28 days after balloon injury; representative samples of n=3, bar=100 μm.
were significantly higher in ApoE<sup>−/−</sup> mice on CD compared with ApoE<sup>−/−</sup> (P<0.05) or WT mice on ND (P<0.01).

**Increased Proliferation and Inflammation in ApoE<sup>−/−</sup> on CD Compared With WT Mice**

In order to get more insight into the kinetics of cell numbers after balloon injury, cell death and proliferation rates were analyzed. Cell death reached around 40% of medial cells at 1 hour postinjury and decreased later on; the differences between the groups were not statistically significant (please see supplemental Figures I and II). In contrast, medial and intimal proliferation rates at 7 and 28 days postinjury (Figure 5A and B) were significantly increased in both ApoE<sup>−/−</sup> on CD compared with ApoE<sup>−/−</sup> on ND as well as ApoE<sup>−/−</sup> on CD compared with WT mice (P<0.05). Neither cell death nor proliferation was detected in untouched control vessels.

Staining for smooth-muscle cells increased 7 days after balloon injury as compared with untouched arteries (P<0.05,
n=3), but injured vessels showed no difference between the groups (Figure 6A). In contrast, intimal leukocytes as well as intimal VCAM-1 expression were both significantly increased in ApoE−/− mice on CD as compared with WT mice (Figure 6B and 6C; P<0.05, n≥3) and untouched control arteries (P<0.001; n=3). These results suggest additive effects of increased plasma cholesterol levels on inflammatory and proliferative responses after balloon injury.

**Discussion**

Using a mouse model of balloon injury, we demonstrate endothelial denudation, platelet activation, early medial cell death, followed by inflammation, proliferation, and neointima. Thus, our model reflects the stages of balloon- or stent-induced lesion formation known from human autopsy and atherectomy series.12,13

By comparing the effects of balloon injury between WT mice on ND, ApoE−/− on ND and ApoE−/− mice on CD, we have detailed the contribution of plasma cholesterol levels on lesion formation. Twenty-eight days after balloon injury, ApoE−/− mice on CD exhibited more pronounced neointima than ApoE−/− or WT mice on a ND. Enhanced vascular lesion formation in hypercholesterolemic ApoE−/− or LDL-receptor−/− mice has been described using a guide wire,14 resin beads,15 cuffs16 or air desiccation combined with stretch.17 However, most of these injury types have limitations with regard to their clinical relevance.

Medial cell death peaked very early after balloon injury and decreased quickly thereafter without significant difference between the groups. These findings match observations in larger rodents.18 The elevated medial and intimal cell proliferation rate in ApoE−/− mice on CD suggests an oxidation-mediated stimulation of medial and intimal smooth-muscle cell proliferation leading to an increased neointima. These findings match data obtained in cell culture and rabbits showing enhanced smooth-muscle proliferation on oxidized low-density lipoprotein (oxLDL).19 Interestingly, we identified the most significant increase in neointimal area and proliferation rates with the addition of dietary cholesterol in ApoE−/− mice. These morphological findings were associated with total plasma cholesterol levels in the different groups. In parallel, Plump and coworkers found a correlation of atherosclerotic lesion formation with total plasma cholesterol levels and addition of dietary cholesterol.4 Although cell death was not significantly different in the 3 groups examined, its early peak after balloon injury is likely to have implications on the following events. Indeed, dying cells may induce a positive feedback and promote—among other factors—the release of inflammatory cytokines.16,20 In accordance with this notion, we observed enhanced recruitment of CD45-positive inflammatory cells and increased expression of VCAM-1 in ApoE−/− mice on CD 7 days after balloon injury. In addition, oxLDL may directly activate the coagulation cascade via activation of tissue factor in cultured smooth-muscle cells21 or induce apoptosis in endothelial cells.22 Thus, balloon angioplasty and oxLDL may induce a direct, combined injury on the vessel wall including endothelial denudation, cell death and activation of coagulation.23 The ensuing “response to injury”9 comprises an inflammatory reaction and proliferation.

We acknowledge that neointima formation in WT mice 28 days after balloon distension was moderate. This is likely attributable to an unopposed healing response in a healthy carotid artery. To increase the response to injury, we recom-
mend to (1) maximize balloon distension, (2) use aspirin via drinking water for decreasing vessel thrombosis, (3) increase plasma cholesterol levels (via CD or selection of an atherogenic genetic background for enhancing the response to injury, and (4) analyze neointima at 14 instead of 28 days.

In summary, we have characterized a novel mouse model of balloon distension injury which imitates the stages of vascular lesion formation induced by percutaneous transluminal angioplasty in the clinical context. ApoE−/− mice on a high cholesterol diet exhibit increased balloon-induced proliferation rates with enhanced neointima formation, in parallel to their elevated total plasma cholesterol levels. Further applications of this miniature balloon in arteries of genetically modified mice will provide attractive opportunities to elucidate molecular mechanisms of vascular injury such as apoptosis, inflammation or proliferation. This know-how may pave the way to catheter-based interventions of human microvessels in the peripheral or cerebral circulation.

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Disclosures
None.
References


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