

# Resistance to Neointimal Hyperplasia and Fatty Streak Formation in Mice With Adrenomedullin Overexpression

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**Objective**—Several in vitro studies have implicated that adrenomedullin (AM) plays an important role in the pathogenesis of vascular injury and fatty streak formation. To test this possibility in vivo, we evaluated 2 experimental models using transgenic mice overexpressing AM in a vessel-selective manner (AMTg mice).

**Methods and Results**—Placement of a periarterial cuff on femoral arteries resulted in neointimal formation at 2 to 4 weeks to a lesser extent in AMTg mice than in their wild-type littermates (at 28 days, intima/media area ratio  $0.45 \pm 0.14$  versus  $1.31 \pm 0.41$ , respectively;  $P < 0.001$ ). This vasculoprotective effect observed in AMTg mice was inhibited by *N*<sup>ω</sup>-nitro-L-arginine methyl ester. We further examined the effect of AM on hypercholesterolemia-induced fatty streak formation by crossing AMTg mice with apolipoprotein E knockout mice (ApoEKO mice). The extent of the formation of fatty streak lesions was significantly less in ApoEKO/AMTg mice than in ApoEKO mice (percent lesion area  $12.0 \pm 3.9\%$  versus  $15.8 \pm 2.8\%$ , respectively;  $P < 0.05$ ). Moreover, endothelium-dependent vasodilatation as indicative of NO production was superior in AMTg/ApoEKO mice compared with ApoEKO mice.

**Conclusions**—Taken together, our data demonstrated that AM possesses a vasculoprotective effect in vivo, which is at least partially mediated by NO. (*Arterioscler Thromb Vasc Biol.* 2002;22:1310-1315.)

**Key Words:** adrenomedullin ■ transgenic mice ■ neointimal hyperplasia ■ fatty streak  
■ apolipoprotein E knockout mice

Many vasoactive factors derived from endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are known to regulate regional vascular tone as well as cellular proliferation and migration, thereby affecting vascular structure. Dysregulated production of these factors is assumed to make a contribution to pathological states, such as hypertension, atherosclerosis, and postangioplasty restenosis.

Adrenomedullin (AM), originally identified as a potent vasodilatory peptide produced by human pheochromocytoma,<sup>1</sup> has been shown to be released from ECs<sup>2</sup> and, to a lesser extent, VSMCs.<sup>3</sup> In addition to its direct<sup>4</sup> and NO-mediated<sup>5</sup> vasodilatory activities, in vitro studies have demonstrated its direct inhibitory effect on the migration<sup>6</sup> and proliferation<sup>7</sup> of VSMCs and its antiapoptotic effect on ECs<sup>8,9</sup> via NO. Therefore, it is suggested that AM has a compensatory effect on deteriorating physiological status and a protective effect against vascular injury as a possible counterpart against vasoconstrictive and mitogenic peptides, including angioten-

sin II and endothelin-1. However, direct evidence for such in vivo roles has still not been well documented.

Recently, we established transgenic mice overexpressing AM (AMTg mice) in a vessel-selective manner driven by the murine preproendothelin-1 promoter.<sup>10</sup> AMTg mice exhibited an increase in AM levels by 2- to 4-fold in the plasma and major organs and by 2- to 8-fold in the aorta by radioimmunoassay. Immunohistochemistry detected intense AM signals in both ECs and VSMCs in the aorta and major arteries as well as in arterioles in vascular-rich organs, including the heart, kidney, and lung. Concerning hemodynamics, chronic AM overexpression in the vessel wall resulted in decreased blood pressure through, at least in part, the stimulation of NO production. We postulated that these mice could also be useful for analyzing the role of AM in the pathophysiological process associated with vascular injury and atherosclerosis. In the present study, we compared the formation of injury-induced neointimal hyperplasia and hypercholesterolemia-

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induced fatty streak formation between mice with and without AM overproduction to clarify whether AM could be involved in the protective mechanisms in the vasculature.

## Methods

The experiments were performed in accordance with the Declaration of Helsinki and the University of Tokyo Institutional Guidelines for Animal Experiments. The mice were housed in an animal room with a 12-hour light-dark cycle and a temperature of 22°C. The mice were given standard chow and water ad libitum, unless otherwise indicated.

## Animals

The AMTg mouse line has already been established as previously described.<sup>10</sup> We crossed AMTg mice with apoE knockout (ApoEKO) mice.<sup>11</sup> To obtain AMTg/ApoEKO mice, homozygous AMTg mice were bred with homozygous ApoEKO mice. The resultant offspring, which overexpressed AM and were obligatorily heterozygous for the mutant apoE locus, were further bred with each other to obtain homozygous ApoEKO mice with or without the AM transgene. The parental ApoEKO mice were F2 hybrids between 129/Sv and C57BL/6, and the parental AM transgenic mice originated from hybrids from the B6C3F1 and C3H strains, which were backcrossed for at least 5 generations into the original background of the ApoEKO mice.

## Hemodynamics

We evaluated blood pressure with a programmable sphygmomanometer (BP98 Softron) by use of the tail-cuff method.<sup>12</sup> All measurements were repeated 10 times for each animal, and the average was obtained. To evaluate the effect of blood pressure reduction, several mice were treated with hydralazine at a dose of 5 mg/kg per day in their drinking water.<sup>13</sup>

## Vascular Injury by Cuff Placement

We used 10- to 12-week-old AMTg and wild-type mice from the same genetic background. The cuff placement surgery was performed according to a method described previously.<sup>14,15</sup> In brief, the mice were anesthetized with ketamine (70 mg/kg) and xylazine (4 mg/kg) by intraperitoneal injection. The left femoral artery was isolated from the surrounding tissues. A polyethylene tube (PE-50, 2 mm long, inner diameter 0.56 mm, outer diameter 0.965 mm; Becton-Dickinson) was cut longitudinally, loosely placed around the artery, and closed with a suture. After the experimental period, the mice were killed, and arterial tissues were fixed in 10% formalin and embedded in paraffin. The middle segment of the artery was cut into 5 subserial cross sections at intervals of 200  $\mu$ m. The sections were stained by elastica van Gieson or hematoxylin and eosin staining. The areas of the neointima, media, and adventitia were measured by using image-analyzing software (NIH image). The average of 5 sections was taken as a representative value for each animal. To evaluate DNA synthesis, bromodeoxyuridine (BrdU, Sigma Chemical Co) was injected at doses of 100 mg/kg SC and 30 mg/kg IP 18 hours before euthanasia and then at a dose of 30 mg/kg IP 12 hours before euthanasia.<sup>16</sup> Immunohistochemistry using anti-BrdU antibody in serial sections was performed (BrdU Staining Kit, Zymed Laboratories), and the BrdU index (the ratio of BrdU-positive nuclei versus total nuclei) was calculated.

To evaluate the chronic inhibition of NO synthase, *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) was dissolved in the drinking water at a concentration of 200 mg/L<sup>17</sup> for 28 days. Moreover, several mice were treated with the AM receptor antagonist CGRP(8-37) to evaluate the effect of AM antagonism. Alzet micro-osmotic pumps (model 1002, Alza) were implanted intraperitoneally at the time of cuff placement. The pumps delivered vehicle (saline) or CGRP(8-37) (20 mg/kg per day, Peptide Institute)<sup>18</sup> continuously for 28 days at a rate of 0.125  $\mu$ L/h.

## Evaluation of Fatty Streak Formation

Two diets were used: (1) a normal chow diet (MF diet from Oriental Yeast Co) that contained 5.6% (wt/wt) fat with 0.09% (wt/wt) cholesterol, and (2) an atherogenic diet, which consisted of the MF diet containing 0.15% (wt/wt) cholesterol and 15% (wt/wt) butter.<sup>19</sup> Eight-week-old ApoEKO/AMTg and ApoEKO mice were fed the atherogenic diet for 2 months and euthanized by an overdose of anesthetic, and the extent of fatty streak formation was evaluated by 2 methods: (1) the en face surface lesion area<sup>20</sup> and (2) the cross-sectional lesion area of aortic origin.<sup>21</sup> In brief, for evaluation of the entire aorta, the aorta from the aortic sinus to the iliac bifurcation was dissected, and lipid-rich atheroma were visualized by staining with Sudan IV. As cross-sectional lesions of the aortic sinuses, 4 serial sections at intervals of 60  $\mu$ m were prepared, stained with oil red O, and counterstained with hematoxylin.

## Vascular Reactivity to Endothelium-Dependent and -Independent Vasodilators

Vascular reactivity to endothelium-dependent and -independent vasodilators was tested by using the descending thoracic aorta to evaluate endothelial function as previously described.<sup>22</sup> The aortic rings were suspended under 1.0 g of tension and precontracted with phenylephrine ( $5 \times 10^{-8}$  mol/L). Acetylcholine (ACh) ( $10^{-9}$  to  $10^{-5}$  mol/L) and sodium nitroprusside (SNP,  $10^{-8}$  to  $10^{-5}$  mol/L) were added cumulatively to the organ bath. For ACh-induced vasodilatation after blockade of NO production, L-NAME ( $10^{-5}$  mol/L) was administered to evaluate endogenous NO production. Data are expressed as the percentage relaxation of phenylephrine-induced precontraction.

## Statistical Analysis

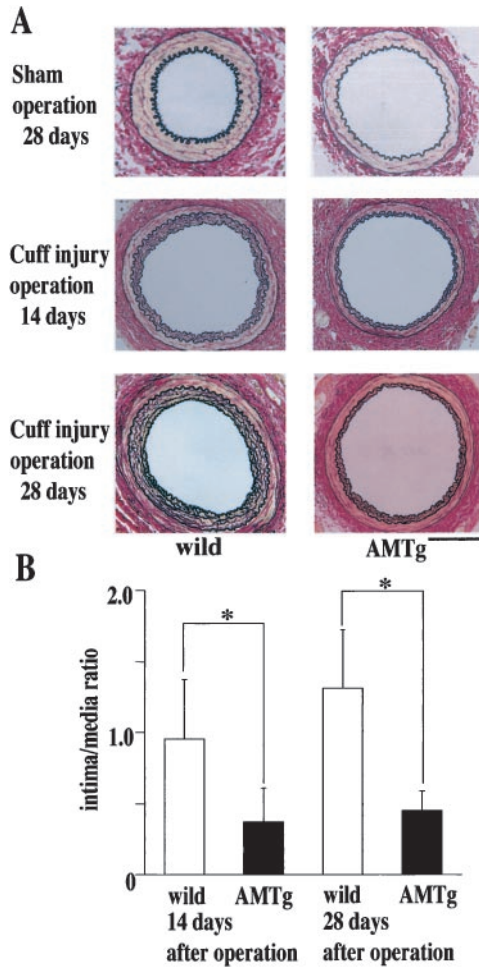
Quantitative values are expressed as the mean  $\pm$  SD. Comparisons of means were made by using the Student *t* test for unpaired values; when >2 means were compared, an ANOVA with repeated measurements was used. If a significant *F* value was found, the Scheffé post hoc test for multiple comparisons was used to identify any differences among groups. A value of *P* < 0.05 was considered significant, unless otherwise indicated. In aortic ring experiments, the negative logarithm of the concentration of ACh or SNP that produced half the putative maximal response was referred to as ED<sub>50</sub>. Maximum vasorelaxation was shown as E<sub>max</sub> (a percentage).

## Results

### Neointimal Formation Induced by Cuff Placement

We compared neointimal formation induced by cuff placement between AMTg and wild-type mice. In contralateral sham-operated arteries, there was no neointima in either the wild-type or AMTg mice (Figure 1A). On the other hand, cuff placement resulted in neointimal hyperplasia, which grew for up to 28 days. Intimal hyperplasia was significantly smaller in AMTg mice than in wild-type mice at 14 days and also at 28 days after surgery (Figure 1A). Twenty-eight days after surgery, the ratio of the neointima area to the intimal/medial area was  $16\,390 \pm 1890 \mu\text{m}^2/1.31 \pm 0.41$  in wild-type mice (*n* = 10) and  $5830 \pm 1620 \mu\text{m}^2/0.45 \pm 0.14$  in AMTg mice (*n* = 10, *P* < 0.001; Figure 1B).

For DNA synthesis of VSMCs, we examined BrdU uptake 3 and 7 days after injury. BrdU uptake in subendothelial layers mainly composed of VSMCs was significantly higher in the wild-type mice (*n* = 7) than in the AMTg mice (*n* = 7). BrdU incorporation was relatively high within 1 week after injury and almost completely diminished  $\geq 14$  days after injury in AMTg and wild-type mice. These data indicate that AM overexpression inhibited the proliferation of VSMCs (Figure 2A).

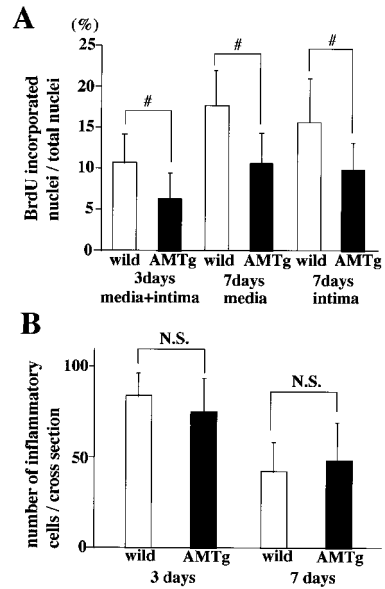


**Figure 1.** A, Photomicrographs showing representative cross sections of the femoral arteries 14 and 28 days after cuff placement. Sections of 5- $\mu$ m thickness were stained by elastica van Gieson staining. B, Morphometric analysis of the cuffed femoral arteries. Cross-sectional areas of media and intima were evaluated at 14 days and 28 days after cuff placement, and the intima/media ratio was calculated from the area of intima divided by the area of media. \* $P$ <0.001.

Inflammatory cell infiltration around the vessels was prominently observed, especially within 1 week after injury. The number of inflammatory cells was not different between AMTg and wild-type mice at 3 and 7 days after injury, indicating that the inflammatory process at the adventitia may not be affected by AM overexpression (Figure 2B).

**Effect of L-NAME, Hydralazine, and CGRP(8-37) on Neointimal Hyperplasia**

The systolic blood pressure measured by the tail-cuff method was significantly lower in the AMTg mice than in the wild-type mice (101.2 $\pm$ 9.3 versus 115.3 $\pm$ 13.2 mm Hg, respectively;  $P$ <0.05). When L-NAME was chronically administered, the systolic blood pressure was significantly elevated, and the difference between the 2 groups diminished (136.5 $\pm$ 7.3 versus 141.2 $\pm$ 11.4 mm Hg for AMTg versus wild-type mice, respectively;  $P$ =NS). For the vascular injury, the vasculoprotective effect in AMTg mice was diminished, and there was no difference in the neointimal response

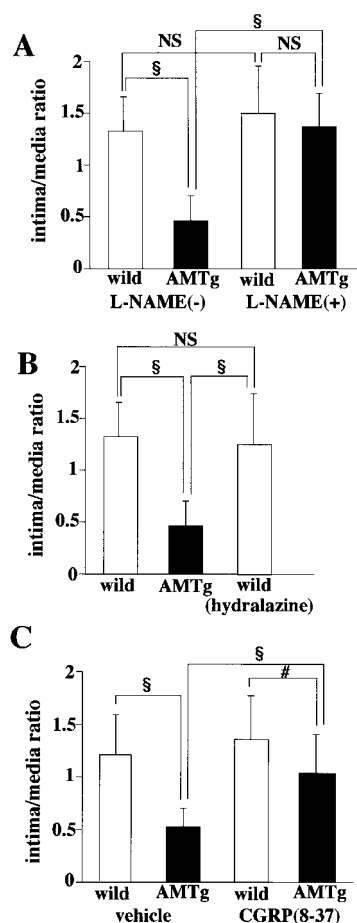


**Figure 2.** A, BrdU uptake in the media and neointima of the cuffed femoral arteries. The BrdU index (the number of BrdU positive nuclei/number of total nuclei) was analyzed 3 and 7 days after cuff placement. B, Number of inflammatory cells invading around injured arteries. The average of 5 sections was taken as the value for each animal. Error bars indicate SD. # $P$ <0.05. NS indicates not significant.

between the AMTg and wild-type littermates (Figure 3A), suggesting that the vasculoprotective action of AM is mediated via an NO-dependent mechanism. Next, to confirm that the vasculoprotective effect of AM is independent of its hypotensive effect, we evaluated the neointimal responses between wild-type mice with and without hydralazine administration. The systolic blood pressure of wild-type mice treated with hydralazine was 98.3 $\pm$ 8.5 mm Hg, which was almost equivalent to that of AMTg mice. We evaluated the neointimal formation 28 days after injury in the wild-type mice treated with hydralazine, finding that the intima/media ratio was 1.25 $\pm$ 0.50, which was not significantly different from that of wild-type mice without hydralazine treatment (1.31 $\pm$ 0.41,  $P$ =NS; Figure 3B). Therefore, the protective effect of AM was independent of the pressure reduction. Moreover, to test the effect of AM antagonism, we treated wild-type mice and AMTg mice with CGRP(8-37) or vehicle. This demonstrated that CGRP(8-37) infusion partially inhibited the beneficial effect of AM overexpression compared with vehicle administration and did not significantly affect neointimal formation in the wild-type mice (Figure 3C).

**Fatty Streak Formation in ApoEKO and AMTg/ApoEKO Mice**

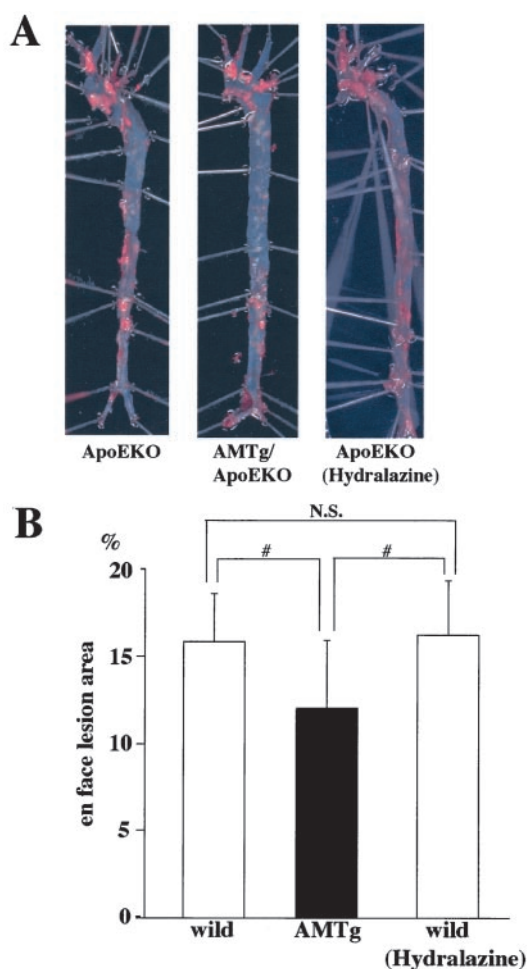
Next, we examined the protective effect of AM on fatty streak formation by breeding AMTg with ApoEKO mice. In AMTg/ApoEKO mice, AM transgene expression was detected in the aorta, heart, lung, and kidney, as evaluated by reverse transcription–polymerase chain reaction and Northern blot analysis (data not shown), and plasma AM levels evaluated by radioimmunoassay<sup>10</sup> were significantly higher in AMTg/ApoEKO mice than in ApoEKO mice



**Figure 3.** Effects of several drug interventions on neointimal formation. A, L-NAME. B, Hydralazine. C, CGRP(8-37). #*P*<0.05 and §*P*<0.01.

(8.36±2.32 versus 4.93±1.07 fmol/mL, respectively; *P*<0.01; n=7 each).

ApoEKO mice and ApoEKO/AMTg mice were fed normal chow until they were 8 weeks old, and then they were fed the atherogenic diet for 2 months. Plasma lipid and lipoprotein concentrations were markedly elevated after an atherogenic diet. However, there were no significant differences in lipid profiles between the AMTg/ApoEKO mice and ApoEKO mice (data not shown). The AMTg/ApoEKO mice (n=16) were significantly more hypotensive than the ApoEKO mice (n=16); systolic blood pressure was 110±15 versus 125±14 mm Hg, respectively (*P*<0.01). Thus, to evaluate the direct effect of hypotension on fatty streak formation, we also evaluated the hydralazine-treated ApoEKO mice, whose systolic blood pressure (104±17 mm Hg) was almost equivalent to that of the AMTg/ApoEKO mice. The representative photographs of en face surface atherosclerotic lesions are shown in Figure 4A. The fatty streak formation of the AMTg/ApoEKO mice (n=12) was significantly smaller than that of the ApoEKO mice (n=12); the en face lesion area was 12.0±3.9% versus 15.8±2.8%, respectively (*P*<0.05, Figure 4B). The same result was observed in the cross-sectional lesion area (223 000±56 000 versus 290 000±45 200 μm<sup>2</sup> for AMTg/ApoEKO mice versus ApoEKO mice, respec-

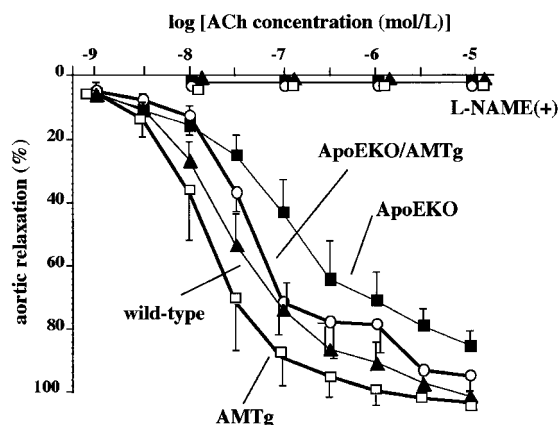


**Figure 4.** Hypercholesterolemia-induced atherosclerotic lesions. A, Representative photographs of en face atherosclerotic lesions in the entire aorta. B, Percentage of en face atherosclerotic lesions. #*P*<0.05.

tively; *P*<0.01). However, the atheromatous lesion formation was not changed in ApoEKO mice when they were treated with hydralazine (en face lesion area 16.2±3.1%, cross-sectional lesion area 301 400±52 100 μm<sup>2</sup>; *P*=NS), suggesting that AM inhibits fatty streak formation via a mechanism other than its hypotensive effect.

**Endothelial Function and NO Production**

To test endothelial function in ApoEKO/AMTg mice and ApoEKO mice, we examined endothelium-dependent and -independent vasodilatation with the use of aortic rings isolated from mice fed the atherogenic diet (n=8 for each group, Figure 5). As controls, wild-type mice and AMTg mice were used for the aortic ring experiment. There was no significant difference in the tension after precontraction by phenylephrine among the 4 groups. The relaxation induced by ACh was significantly deteriorated in the apoE-deficient mice compared with the AMTg and wild-type mice. However, endothelium-dependent vasorelaxation was significantly better in ApoEKO/AMTg mice (*E*<sub>max</sub> 94.6±2.5%, *ED*<sub>50</sub> [-log molar] 7.31±0.14) than in ApoEKO (*E*<sub>max</sub> 85.2±4.6%, *ED*<sub>50</sub> 6.83±0.25; *P*<0.05). Concerning the 2 control groups, the



**Figure 5.** Endothelium-dependent vasorelaxation of aortas from the 4 groups: wild-type (closed triangle), AMTg (open square), ApoEKO (closed square), and AMTg/ApoEKO (open circle) mice ( $n=8$  to 10). Values are mean  $\pm$  SEM.  $\#P<0.05$ .

ED<sub>50</sub> was also significantly better in AMTg mice ( $7.39 \pm 0.30$ ) than in wild-type mice ( $7.77 \pm 0.16$ ). However, there was no significant difference in E<sub>max</sub> between AMTg mice ( $97.7 \pm 2.4\%$ ) and wild-type mice ( $99.7 \pm 0.4\%$ ,  $P=NS$ ). On the other hand, there was no significant difference in aortic relaxation in response to SNP among the 4 groups. ACh-induced relaxation was almost completely attenuated by L-NAME, suggesting that the vasorelaxation induced by ACh is almost exclusively mediated by endothelium-derived NO. These data suggest that endothelial function was preserved by AM overexpression when damaged by remarkable hypercholesterolemia. Moreover, plasma cGMP levels were also significantly higher in AMTg/ApoEKO mice ( $7.9 \pm 2.0$  pmol/mL) than in ApoEKO mice ( $6.1 \pm 1.8$  pmol/mL,  $P<0.05$ ), implicating that the steady-state NO production was also upregulated in mice with AM overexpression. On the other hand, the endothelial NO synthase (eNOS) expression levels in aorta detected by reverse transcription–polymerase chain reaction were not different between mice with and without AM overexpression (data not shown), suggesting that the function of eNOS may be regulated by AM at the posttranslational or protein levels.

## Discussion

In the present study, we examined the involvement of AM in the formation of vascular lesions by analyzing 2 different experimental models. We evaluated neointimal formation induced by cuff placement and hypercholesterolemia-induced fatty streak formation and found that mice carrying AM transgenes were significantly resistant to vascular injury and fatty streak formation. This is the novel important evidence that supports the vasculoprotective effect of AM *in vivo*.

Rodents are naturally resistant to neointimal hyperplasia and fatty streak formation. Therefore, several experimental models of neointimal hyperplasia or atherosclerosis have been proposed. In the present study, we adopted the cuff-injury model, in which the endothelial layer remains intact and thrombus formation is very rare because no intravascular manipulation is performed. Using this model, we demonstrated that AM overexpression suppressed neointimal hyper-

plasia and that BrdU-positive nuclei in the subendothelial area were reduced in the cuff-injured femoral arteries, indicating that the proliferation of VSMCs was downregulated by AM. This evidence is consistent with previous *in vitro* experiments.<sup>7</sup> On the other hand, there was no significant difference in inflammatory cells around the injured arteries between AMTg and wild-type mice, suggesting that inflammation may not be affected by AM overproduction.

AM can act on endothelial cells to stimulate NO production via calcium-dependent activation of eNOS.<sup>5,23</sup> We tested whether inhibition of NO production diminished the protective effect of AM. Chronic administration of L-NAME abolished the beneficial effect of AM overexpression on vascular injury. Therefore, the vasculoprotective effect of AM was thought to be at least partially mediated by NO. The beneficial effect of NO produced by endothelium has been previously demonstrated in many reports. eNOS knockout mice have been reported to exhibit abnormal intimal hyperplasia after vascular injuries in the cuff placement.<sup>14</sup> eNOS overexpression has also been shown to inhibit neointimal formation significantly in the rat carotid balloon injury model.<sup>24</sup> These previous studies and the present result lead us to postulate that the activation of eNOS by AM may be effective in preventing the formation of vascular proliferative lesions.

Shimizu et al<sup>18</sup> reported that the AM antagonist CGRP(8-37) inhibits neointimal hyperplasia after balloon injury in the rat carotid artery, indicating that endogenous AM in the injured tissue may promote the proliferation of VSMCs. This observation seems to contradict our data. In the present study, AM overexpression inhibited neointimal formation, and CGRP(8-37) infusion partially inhibited the beneficial effect of AM overexpression. This discrepancy may be due to differences in the experimental animals and systems. In our 2 models, the endothelial layers remained intact, and AM was overexpressed mainly in the endothelium. On the other hand, in a rat carotid artery balloon-injury model, the endothelial layers were denuded, and the beneficial effect of AM on endothelial layers was abolished. The injured VSMCs were exposed directly to the blood stream; thus, the phenotypic changes in VSMCs might occur more easily. Concerning other type of cells, AM has an antiproliferative effect of glomerular mesangial cells<sup>25</sup> but stimulates cell proliferation in Swiss 3T3 cells.<sup>26</sup> Therefore, it is likely that the effect of AM on cell proliferation depends on the phenotypes or experimental situations.

To date, apoE knockout mice have been useful in the analysis of cholesterol-induced fatty streak formation. Several experiments on crossbreeding with other genetically altered mice or on pharmacological intervention in ApoEKO mice have been reported.<sup>19,27</sup> Thus, we crossbred AMTg mice with ApoEKO mice to clarify the effect of AM on hypercholesterolemia-induced fatty streak formation. We demonstrated that the presence of AM transgenes suppresses the formation of atheromatous lesions and that this is independent of the blood pressure reduction and lipoprotein profiles. It is worth noting that the vasculoprotective effect of AM was also confirmed in this model.

Endothelial dysfunction is characterized by impaired endothelium-derived NO-mediated vasorelaxation in atherosclerosis in humans and experimental animals. A previous report<sup>28</sup> demonstrated that endothelial dysfunction was impaired in isolated aortic rings of ApoEKO mice compared

with wild-type mice, which was replicated in the present study. In the present study, we found that endothelium-dependent vasodilatation was superior in AMTg/ApoEKO mice compared with ApoEKO mice, although severe dyslipidemia damaged endothelium-dependent vasorelaxation significantly. Moreover, the plasma cGMP level (taken to be indicative of steady-state NO production) was significantly higher in ApoEKO/AMTg mice than in ApoEKO mice. Therefore, our data suggest that the antiatherogenic effect of AM in ApoEKO is at least partially caused by endothelium-derived NO production by chronic AM overexpression.

In summary, we demonstrated the beneficial effects of AM in vivo against vascular injury at least partially via endothelial NO production with the use of genetically altered mice. However, further studies are needed to clarify the precise mechanism of the vasculoprotective effect of AM so that AM can be applied clinically for atherosclerosis and for postangioplasty neointimal hyperplasia.

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