Adrenomedullin Overexpression to Inhibit Cuff-Induced Arterial Intimal Formation

Masao Yamasaki, Junsuke Kawai, Takashi Nakaoka, Teruhiko Ogita, Akihiro Tojo, Toshiro Fujita

Abstract—Adrenomedullin (AM) inhibits vascular smooth muscle cell proliferation stimulated by fetal calf serum and platelet-derived growth factor in vitro. In this study, an adenovirus expressing AM (AxCAAM) was created to examine the in vivo action of AM. Femoral arteries of Wistar rats were wrapped with a silicone cuff and treated with adenovirus expressing Escherichia coli β-galactosidase (AxCALacZ) or AxCAAM. Immunoreactivity for endothelial nitric oxide synthase (eNOS) was reduced in the endothelium of cuff-injured arteries and was associated with increased local DNA synthesis. Consequently, the intimal formation measured by the intimal-to-medial ratio was significantly increased at 14 and 28 days after the cuff placement. AxCAAM-infected arteries increased the expression of eNOS in the endothelium and inducible NOS in the media and the adventitia. AxCAAM significantly decreased the intimal-to-medial ratio by 40% at 14 days and 51% at 28 days, whereas AxCALacZ showed no changes compared with cuff-injured control arteries. AM overexpression effectively limits intimal hyperplasia by reducing cell proliferation through a nitric oxide–dependent pathway of eNOS. Our findings suggest the possibility of a therapeutic use of the AM gene for the prevention of vascular proliferative disorders. (Hypertension. 2003;41:302-307.)

Key Words: adrenomedullin ■ muscle, smooth, vascular ■ endothelium ■ nitric oxide synthase

Adrenomedullin (AM) was first isolated from the acid extract of human pheochromocytoma as a potent hypotensive peptide.1 This peptide, consisting of 52 amino acids, has been shown to have several actions other than its vasodilatory effect. AM inhibits the proliferation and migration of cultured rat aortic smooth muscle cells (SMCs) and rat mesangial cells.2–4 This peptide stimulates cAMP formation in cultured cells;2,3 however, some actions are not solely through the elevation of intracellular cAMP. AM acts not only on cells of mesenchymal origin such as SMC or mesangial cells but also on other cell types. It was demonstrated that AM stimulates the proliferation of fibroblasts and certain tumor cell lines.5 Therefore, it seems that the effect of AM on mitogenesis might depend on the particular cell type. Considering that the actions of AM are so diverse, it is very important to examine the effect of AM in the setting of multicellular circumstances such as in vivo models to further elucidate the actions of AM.

Intimal thickening is an early, essential stage in the development of atherosclerotic lesions. In experimental animals, several models have been established to mimic this pathologic condition and to assess the efficacy of therapeutic strategies. Intimal thickening can be induced by balloon denudation of the endothelium, by ligation of the vessel, or by electrical injury. Recently, it has been reported that placement of a nonconstrictive cuff around an arterial segment in rodents results in a reproducible, concentric intimal hyperplasia within 14 days.7–10 Intimal thickening induced by these techniques results from the excessive accumulation of SMC and the deposition of extracellular matrix in the intimal layer of the vessel wall. The cellular component of the intima predominantly consists of α-smooth muscle actin–positive cells. Because adenovirus-mediated gene transfer into an arterial segment from the outside is reportedly feasible,11 we sought to examine the effect of the adenovirus-mediated gene transfer of AM on the cuff-induced intimal thickening of rat femoral artery. In addition, the expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in the cuff-injured artery were examined as well as the cAMP formation in this study, since there is a possible involvement of nitric oxide (NO) production in the prevention of atherosclerosis.

Methods

Animals

Male Wistar rats (Nippon Bio-Supply Center, Tokyo) weighing 350 to 400g were used in this study. They were kept individually in stainless steel cages in a room where lighting was controlled (12 hours on, 12 hours off) and the temperature was kept at ~22°C. They were given standard diet (MF, Oriental Yeast Co, Ltd) and water ad libitum. All procedures were performed under sterile conditions according to the Guide for Animal Experimentation, Faculty of Medicine, University of Tokyo.

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Materials
A replication-defective adenovirus carrying the Escherichia coli β-galactosidase gene, AxCALacZ, was kindly provided by Dr I. Saito (University of Tokyo, Japan) and human AM cDNA by Dr N. Minamino and Dr K. Kangawa (National Cardiovascular Center Research Institute, Osaka, Japan). The adenovirus expression kit was purchased from Takara Co, bromodeoxyuridine (BrdU) from Sigma Chemical Co, monoclonal mouse anti-BrdU antibody from DAKO Co, both cAMP and cGMP radioimmunoassay kit from Yamasa Co, and rabbit polyclonal antibody against eNOS and iNOS from Transduction Laboratories. Monoclonal mouse anti-human AM amidated C-terminal peptide (aa 46 to 52) antibody (anti-AM) was described previously.13,14

Construction of an Adenovirus Vector Carrying AM Gene
To examine the effect of adenovirus-mediated gene transfer of AM on injury-induced arterial remodeling, we constructed a replication-defective adenovirus carrying the AM gene under CAG promoter (AxCAAM). A full-length coding region of AM was blunt-ended and cloned into a cosmids, pAXCAwt (adenovirus expression kit). The resulting cosmid, pAXCAAM, was cotransfected into the 293 embryonic cell line with EcoT221-digested DNA-TTP (from Ad5dlx) to generate AxCAAM, carrying the coding region of AM. The obtained viruses were isolated, screened for the AM insert, and propagated. For the in vivo experiments, the viruses were purified and stored in PBS containing 10% glycerol at −80 °C.

Adenovirus-Mediated Gene Transfer Into Cuffed Arteries
The surgical procedures of cuff placement were applied according to the method described previously, with some modification.8 Rats were anesthetized with 45 mg/kg IP sodium pentobarbital. The femoral artery was isolated from the surrounding tissues, and a silicone rubber tube (5 mm long; inner diameter, 0.64 mm; outer diameter, 1.20 mm; Plastics One), cut longitudinally to open the tube, was loosely placed around the artery. Simultaneously, a 6-mm diameter, 1.20 mm; Plastics One) cuff was wrapped around the femoral artery, removed 2, 5, and 14 days after the adenoviral infection, was fixed and paraffin-embedded, and rabbit polyclonal antibody against eNOS and iNOS from Transduction Laboratories. Monoclonal mouse anti-human AM amidated C-terminal peptide (aa 46 to 52) antibody (anti-AM) was described previously.13,14

Immunostaining of AM and X-Gal Staining
To examine the AM expression, a portion of the femoral artery, removed 5 and 14 days after the adenoviral infection, was fixed in PBS containing 4% paraformaldehyde for 2 hours at room temperature and then paraffin-embedded. Four-micrometer sections from each block of arterial tissue were stained immunohistochemically by the avidin-biotin complex method, with the use of an ABC kit and antibodies against AM, eNOS, and iNOS. Areas of cuff-injured arteries at 5 days after cuff placement, were weighed (≈1 mg) and homogenized in ice-cold 6% TCA. After centrifuging, supernatant was extracted in water-saturated diethyl ether, and both cAMP and cGMP content were determined with a commercially available [125I]-cAMP and [125I]-cyclic GMP radioimmunoassay kit.

Statistical Analysis
All values are expressed as mean ± SEM. Statistical analysis was performed by 1-way ANOVA followed by the Fisher post hoc test. Differences at a level of P < 0.05 were considered to be statistically significant.

Results
Adenovirus-Mediated Gene Transduction Into Cuff-Injured Arteries
We assessed the AM expression in cuff-injured artery by immunohistochemistry. A slightly increased AM staining was detected in perivascular cells in the cuff-injured artery at 5 days after cuff placement, as compared with that in the native intact artery (Figures 1a and 1b). Perivascular cells of AxCAAM-infected arteries at 5 days after cuff placement showed a prominent reactivity for AM (Figure 1c). This reactivity for AM was further increased in the intima, media, and adventitia of AxCAAM-infected cuff-injured artery harvested 14 days later (Figure 1d). A portion of the cuff-injured artery, infected with AxCALacZ, was stained with AM antibodies and X-gal to further confirm the adenovirus-mediated gene expression by this method. As a result, perivascular cells at 5 days after cuff placement expressed a staining with AM comparable to that of the cuff-injured artery (Figure 1e) and intense β-galactosidase activity (Figure 1f), as in a previous report.11

Suppression of DNA Synthesis by AxCAAM
We assessed the DNA synthesis of the medial cells at 2, 5, and 14 days by BrdU labeling of the nuclei of the cells after cuff injury. Increased BrdU-positive nuclei were detected in
medial cells in the cuff-injured artery infected with AxCA-LacZ. When the cuff-injured artery was infected with AxCAAM, BrdU-positive nuclei were significantly decreased compared with AxCALacZ-infected artery at every time point (Figure 2). Therefore, the decrease in DNA synthesis in AxCAAM-infected medial cells probably was due to the overexpression of AM.

AxCAAM Limits Cuff-Induced Intimal Formation

A rat cuff-injury model is a well-established system to examine vascular smooth muscle cell (VSMC) proliferation. Intimal hyperplasia developed in the femoral artery 5 days after a silicone cuff was placed around the artery. The intimal area of the sections from cuff-injured artery was $7.0 \pm 10^{-2}$ mm$^2$ at 14 days and $7.5 \pm 10^{-2}$ mm$^2$ at 28 days after cuff placement. The I/M ratio of the cuff-injured artery was 22.3% at 14 days and 16.7% at 28 days after injury. The slightly decreased value at 28 days probably was due to an expansion of the medial mass.

To address the effect of AM gene transduction on the cuff-injured artery, arteries were infected either with AxCAAM or with AxCALacZ. The arteries were harvested for histological analysis at 14 and 28 days after cuff placement. The infection of AxCAAM significantly decreased intimal formation (I/M ratio: 13.4% at 14 days, 8.1% at 28 days). In contrast, intimal formation in the cuff-injured artery infected with AxCALacZ was comparable to that of arteries not infected with the virus both at 14 and 28 days after injury (Figure 3). There was no difference in medial mass between the groups. The I/M ratio of AxCAAM-infected artery was comparable to that of sham-operated control (I/M ratio, 6.7% at 28 days). Thus, an adeno virus encoding the AM gene can effectively limit intimal hyperplasia after cuff injury. Figure 4 shows representative histological photomicrographs of cross sections of femoral arteries at 14 and 28 days after cuff placement.

Figure 2. Bar graphs show BrdU labeling index of medial cells, determined as the ratio of BrdU-positive nuclei of medial cells to the total number of medial cells. Transfection of AxCAAM significantly decreased BrdU labeling index as compared with that of the cuff-injured artery transfected with AxCALacZ at 2, 5, and 14 days after cuff placement. Data are mean±SEM. Native indicates native intact artery; Sham, sham-operated artery; Cuff-LacZ, cuff-injured artery transfected with AxCALacZ; and Cuff+AM, cuff-injured artery transfected with AxCAAM. n=4 to 5 in each group. #P<0.0001 vs sham; *P<0.0001 vs Cuff+LacZ.

Figure 3. Bar graphs show I/M area ratio at 14 and 28 days after cuff placement. Transfection of AxCAAM significantly decreased I/M area ratio both at 14 and 28 days after cuff placement, but AxCALacZ did not suppress neointimal formation of cuffed arteries. Data are mean±SEM. Sham, Cuff+LacZ, and Cuff+AM are the same as described in Figure 2. Cuff indicates cuff-injured artery. n=5 to 7 in each group. #P<0.05 vs sham; ##P<0.001 vs sham; *P<0.05 vs Cuff+LacZ; **P<0.001 vs Cuff+LacZ.
Moreover, AM overexpression could significantly increase by AM in intimal thickening. Further studies are necessary to clarify the role of NO-dependent pathway of eNOS. However, the role of increased iNOS in the media and adventitia might be due to the endothelial damage by intimal thickening and to the eNOS suppression by the increased NO from iNOS in the VSMCs. The expression of eNOS in endothelium and iNOS in the media and in the adventitia were markedly enhanced by AM gene transfection in the cuff-injured artery, associated with the increased AM expression in the endothelial cells and in the adventitia. Uregulated expression of eNOS and iNOS by AM gene transfection might produce enough amounts of NO to inhibit the proliferation of VSMC and to inhibit the DNA synthesis suppressing neointimal formation. Thus, AM gene transfection in the cuff-injured artery might inhibit intimal thickening through an NO-dependent pathway of eNOS. However, the role of increased iNOS in the media and the adventitia remained unclear. Further studies are necessary to clarify the role of NO increase by AM in intimal thickening.

In the present study, we showed that adenovirus expressing the AM AxCAAM inhibited intimal formation in a cuff-injury model. AM belongs to the CGRP family. At present, it is believed that AM shares its receptor with CGRP, whereas there are other sole receptors for AM, such as receptor
activity-modifying protein (RAMP)-2 or 3. It was reported that the peptide corresponding to aa8-37 of CGRP (CGRP8-37), an AM receptor antagonist, could inhibit carotid artery intimal formation in a rat balloon injury model. We examined the effect of CGRP8-37 on intimal formation in a cuff-injury model by using a miniaturized osmotic pump according to the described method, with some modification. In the present study, CGRP8-37 was continuously infused after cuff placement at 25 pmol/kg per minute by a miniaturized osmotic pump implanted in abdominal subcutaneous pockets until 14 days later, and 60 nmol/kg SC bolus injections were made once daily for 3 days, commencing immediately after cuff injury. However, the intimal formation was not affected by the administration of CGRP8-37 (data not shown). Accordingly, the discrepancy between the results of the two studies might be due to differences between the two models. First, it is possible that the difference is or is not caused by the existence of endothelium, since AM was shown to release NO from the endothelium. However, it should be noted that eNOS expression was apparently reduced even in endothelial cells of cuff-injured artery. Second, the intimal formation in cuff-injured artery is slower and less intense than that in balloon-injured artery, raising the possibility that the cellular process for the formation of the intima in cuff injury might differ from that in balloon injury. Third, the intimal cells in the balloon injury model showed marked AM immune reactivity, whereas AM expression was not detected in the intimal cells in the cuff-injury model despite the increased AM staining in the perivascular cells. Therefore, the discrepancy might be attributable to differences in the distribution of CGRP receptors and exclusively AM receptors between intimal cells in the balloon-injured artery and perivascular cells in the cuff-injured artery. However, the results of our study are thoroughly supported by those of sophisticated experiments with the method of gene manipulation, which showed that cuff-induced intimal hyperplasia was markedly suppressed in AM-overexpressing transgenic mice and was apparently augmented in AM-knockout mice (data not shown). These results therefore strongly suggest that AM has the ability to inhibit cuff-induced intimal hyperplasia.

In this model, the silicon cuff would work as a foreign body that induces an immune response and inflammation. In several vascular injury models, inflammation is reported to play a critical role in forming vascular lesions. Recent data indicate that inflammatory cells can release enzymes that generate angiotensin II (AngII), including ACE and chymase. Tissue ACE and chymase produced within an atherosclerotic lesion contribute to high local levels of AngII, leading to the progression of lesion formation via the proliferation of SMCs. Of note, several investigators have reported a marked accumulation of tissue ACE and AngII in balloon-injured and cuff-injured arteries. Actually, both ACE inhibitors and angiotensin type 1 (AT1) receptor antagonists reduce intimal lesion formation after balloon injury. Thus, the importance of the local renin-angiotensin system in SMC proliferation and migration after both balloon injury and cuff injury is widely accepted. Whereas AngII stimulates the migration of rat aortic SMCs, AM has an antagonistic action against AngII-induced cell proliferation and migration in VSMCs and mesangial cells. This was supported by the result of our recent study showing that AM knockout mice had more severe intimal hyperplasia and perivascular fibrosis of coronary arteries induced by the treatment of AngII and salt loading as compared with wild mice. In the present study, therefore, the AM overexpression–induced inhibition of arterial intimal hyperplasia might be mediated partly by the protective action of AM against AngII, the formation of which was locally stimulated by the cuff injury.

**Perspectives**

Since the phenotypic changes of SMCs are involved in the development of arterial intimal thickening induced by balloon injury, it will be interesting to examine the phenotypic change of these cells exposed to AM. If AM contributes to maintaining the vascular structure by preventing SMCs from switching to a cell cycle–prone phenotype, AM could be a favorable candidate for the prevention of cardiovascular diseases.

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**References**


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