Adrenomedullin Gene TransferInduces TherapeuticAngiogenesis in a Rabbit Model of Chronic HindLimb IschemiaBenefits of a Novel Nonviral Vector, Gelatin

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Background—Earlier studies have shown that adrenomedullin (AM), a potent vasodilator peptide, has a variety ofcardiovascular effects. However, whether AM has angiogenic potential remains unknown. This study investigatedwhether AM gene transfer induces therapeutic angiogenesis in chronic hind limb ischemia.

Methods and Results—Ischemia was induced in the hind limb of 21 Japanese White rabbits. Positively chargedbiodegradable gelatin was used to produce ionically linked DNA-gelatin complexes that could delay DNA degradation.Human AM DNA (naked AM group), AM DNA-gelatin complex (AM-gelatin group), or gelatin alone (control group)was injected into the ischemic thigh muscles. Four weeks after gene transfer, significant improvements in collateralformation and hind limb perfusion were observed in the naked AM group and AM-gelatin group compared with thecontrol group (calf blood pressure ratio: 0.60±0.02, 0.72±0.03, 0.42±0.06, respectively). Interestingly, hind limbperfusion and capillary density of ischemic muscles were highest in the AM-gelatin group, which revealed the highestcontent of AM in the muscles among the three groups. As a result, necrosis of lower hind limb and thigh muscles wasminimal in the AM-gelatin group.

Conclusions—AM gene transfer induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia.Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhancedthe therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategyfor the treatment of peripheral vascular diseases. (Circulation. 2004;109:526-531.)

Key Words: peripheral vascular disease ■ angiogenesis ■ gene therapy ■ ischemia

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.1 AM and its receptor are expressed mainly in vascular endothelial cells and vascular smooth muscle cells.2-4 AM not only induces vasorelaxation but also regulates growth and death of these vascular cells.5-10 These findings suggest that AM plays an important role in maintaining vascular homeostasis in an autocrine and/or paracrine manner.

A recent study has shown that vascular abnormalities are present in homozygous AM knockout mice, suggesting that AM is indispensable for vascular morphogenesis.11-13 More recently, AM has been shown to activate the PI3K/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.7,14 These results raise the possibility that AM plays a role in modulating vasculogenesis and angiogenesis. However, whether AM induces therapeutic angiogenesis remains unknown.

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526
We prepared biodegradable gelatin that could hold negatively charged protein or plasmid DNA in its positively charged lattice structure. Biodegradable gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation. Similarly, ionically linked DNA-gelatin complexes can delay gene degradation. These findings raise the possibility that gelatin may serve as a nonviral vector for gene therapy.

Thus, the purposes of this study were (1) to investigate whether AM gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia and (2) to examine whether the use of biodegradable gelatin as a vector augments AM expression and thereby enhances the therapeutic effects of AM gene transfer.

Methods

Animal Model
All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Twenty-one male Japanese White rabbits (body weight, 2.9 ± 0.1 kg; Japan Animal Co, Osaka, Japan) were used for physiological and morphological assessment. In addition, 30 rabbits were used for radioimmunoassay, immunohistochemical examination, and Western blot analysis. After anesthetization with pentobarbital sodium (30 to 35 mg/kg), a longitudinal incision was made in the left thigh, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Hind limb ischemia was induced by ligation of the distal left external iliac artery and complete resection of the left femoral artery, as described previously.

Construction of Plasmid DNA
To construct the expression vector for human AM, the EcoRI/XhoI fragment of the full-length human AM cDNA was ligated into the EcoRI/XhoI fragment of the pcDNA1.1-CMV expression plasmid (Invitrogen). To verify that the pcDNA1.1-CMV vector encoding AM cDNA produces a biologically active AM protein, the expression vector was transfected into 293 cells, and AM activity in the transfected cells was measured by high-performance liquid chromatography and radioimmunoassay. The pcDNA1.1-CMV vector encoding β-galactosidase (LacZ) cDNA was used as a control DNA.

Preparation of AM DNA-Gelatin Complex
Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of approximately 30 μm, water content of 95%, and an isoelectric point (pI) of 9 after swelling in water. Gelatin can hold negatively charged protein or plasmid DNA in its positively charged lattice structure (Figure 1A). Dried gelatin (4 mg, pI 9) was added to human AM DNA solution (500 μg/100 μL in phosphate-buffered saline, pH 7.4). After mixture of DNA and gelatin, DNA-gelatin complexes were incubated at 37°C for 2 hours.

To visualize incorporation of DNA into gelatin, AM plasmid DNA was labeled with rhodamine B isothiocyanate (RITC), as reported previously. In brief, the coupling reaction of RITC to plasmid DNA was carried out by mixing the two substances in 0.2 mol/L sodium carbonate-buffered solution (pH 9.7), followed by gel filtration with a PD 10 column (Amersham-Pharmacia). RITC-labeled AM DNA was incorporated into positively charged gelatin (Figure 1B).

Study Protocol
Ten days after the induction of hind limb ischemia (day 10), AM DNA (naked AM group, n = 7), AM DNA-gelatin complex (AM-gelatin group, n = 7), or gelatin alone (control group, n = 7) was administered intramuscularly into 3 different sites in the ischemic adductor muscle and 2 different sites in the semimembranosus muscle. In addition, Lac Z DNA-gelatin complex served as a control DNA (Lac Z-gelatin group, n = 5). The amount of plasmid was 500 μg (1 mL) and that of gelatin was 4 mg. Morphological and angiographic analyses and measurements of calf blood pressure and laser Doppler flow were performed 4 weeks after gene transfer (day 38). After completion of these measurements, the adductor, semimembranosus, and gastrocnemius muscles were weighed in each hind limb. The muscle weight ratio was calculated for each muscle as follows: muscle weight ratio = muscle weight in ischemic hind limb/muscle weight in nonischemic hind limb. Specimens of the adductor muscle of the ischemic hind limb were obtained for histological examination.

Measurement of Calf Blood Pressure
Calf blood pressure was measured on days 10 and 38 in both hind limbs with a Doppler flowmeter (Hayashi Denki Co, Ltd) and a 25-mm-wide cuff. The pulse of the posterior tibial artery was identified with the use of a Doppler probe, and the systolic blood pressure in both hind limbs was determined by standard techniques. The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic hind limb to that of the normal hind limb.

Laser Doppler Blood Perfusion Analysis
Blood flow of the ischemic hind limb was measured with the use of a laser Doppler blood perfusion image system (moorLDI, Moor Instruments) on day 38.

Angiographic Analysis
Development of collateral arteries was evaluated by angiography on days 0 and 38. A 4F catheter was placed in the left internal iliac artery through the common carotid artery, and 3 mL contrast medium (Iopamiron 300, SCHERING) was injected with an automated angiography injector at a rate of 2.5 mL/s. Quantitative angiographic analysis of collateral vessel development in the ischemic hind limb was performed with the use of a 5-mm² grid overlay, as described previously. The angiographic score was calculated for each film as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the ischemic medial thigh. The angiographic score was determined by 2 blinded observers.
Morphological and Histological Examination
The degree of lower hind limb necrosis and thigh muscle necrosis was macroscopically evaluated on graded morphological scales (grade 1 to 3) for peripheral tissue damage and muscle necrosis area of the adductor, semimembranous, and medial large muscles. Capillary density of the ischemic hind limb was evaluated by alkaline phosphatase staining, as reported previously. A total of 10 different fields from three different sections were randomly selected, and the number of capillaries was counted under a ×40 objective. Capillary density was expressed as the mean number of capillaries per square millimeter. The number of myofibers in each field was also examined and the capillary/muscle fiber ratio calculated.

Radioimmunoassay for Human AM
Human AM production was examined 1, 2, and 4 weeks after gene transfer in the naked AM group, AM-gelatin group, and control group (n = 5 each). The muscles were harvested for radioimmunoassay and immunohistochemical examination. Immunoreactive human AM level in rabbit muscles was determined by immunoradiometric assay with the use of a specific kit (Shionogi Co, Ltd). Tissue content of vascular endothelial growth factor (VEGF) was examined by ELISA kit (R&D systems).

Immunohistochemistry for Human AM, Ki67 Antigen, and Phosphorylated Akt
Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded 4-μm sections of ischemic thigh muscles 7 days after gene transfer. To elucidate AM expression after gene therapy, immunohistochemistry for human AM was performed with the use of a monoclonal antibody recognizing AM-(12–25) (1:100), as reported previously. To evaluate the proliferative potential of AM, tissue sections were stained for Ki67, a marker for cell proliferation, with the use of monoclonal anti-Ki67 antibody (1:100) (DAKO). AM has recently been shown to promote proliferation of vascular endothelial cells at least in part through the PI3k/Akt pathway. Thus, immunohistochemistry for phosphorylated Akt was performed with mouse monoclonal anti-phosphorylated Akt antibody (1:100) (Cell Signaling Technology).

Western Blot Analysis
To identify Akt phosphorylation in ischemic muscles after AM gene transfer, Western blotting was performed with the use of a commercially available kit (PhosphoPlus Akt [Ser473] Antibody Kit, Cell Signaling Technology). Ischemic muscles in the 3 groups were obtained 7 days after AM gene transfer. These samples were homogenized on ice in 0.1% Tween 20 homogenization buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 minutes at 4°C, the supernatant was used for Western blot analysis. The 50 μg of protein was transferred into sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membrane through the use of a wet blotting system. After blocking for 60 minutes, the membranes were incubated with primary antibodies (1:500) at 4°C overnight. The membranes were then incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Cell Signaling Technology), at a final dilution of 1:2000. Signals were detected through the use of LumiGLO chemiluminescence reagents (Cell Signaling Technology).

Statistical Analysis
All results are expressed as mean±SEM. Statistical significance was evaluated by 1-way ANOVA followed by Fisher’s analysis, Scheffe’s F analysis, or Kruskal-Wallis test. A value of P<0.05 was considered statistically significant.

Results
Physiological and Morphological Assessment
Complete resection of the left femoral artery resulted in a similar decrease in calf blood pressure ratio among the 3 groups before the initiation of therapy (day 10) (Figure 2A). However, the calf blood pressure ratio on day 38 was highest in the AM-gelatin groups, followed by the naked AM group and subsequently the control group. The laser Doppler flow in hind limb was highest in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2B). The calf blood pressure ratio and laser Doppler flow 4 weeks after gene transfer did not significantly differ between the control group and Lac Z-gelatin group. Lower hind limb necrosis was minimal in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2C). Thigh muscle necrosis was also minimal in the AM-gelatin group. Similarly, the muscle weight ratio (ischemic/normal) on day 38 was highest in the AM-gelatin group (Table). Neither mean arterial pressure nor heart rate significantly differed among the 3 groups.

Angiographic Analysis
Angiograms 4 weeks after gene transfer (day 38) showed the development of collateral arteries in the naked AM and

**Physiological Characteristics**

<table>
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<tr>
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<th>Control</th>
<th>Naked AM</th>
<th>AM-Gelatin</th>
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<tbody>
<tr>
<td>No. of rabbits</td>
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<tr>
<td>Body weight, kg</td>
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<td>114±3</td>
<td>116±2</td>
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<tr>
<td>HR, beats/min</td>
<td>269±12</td>
<td>253±5</td>
<td>262±7</td>
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<tr>
<td>Muscle weight ratio</td>
<td>0.71±0.03</td>
<td>0.84±0.02*</td>
<td>0.95±0.02†</td>
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MAP indicates mean arterial pressure; HR, heart rate; and muscle weight ratio, ratio of muscle weight in ischemic hind limb to that in nonischemic hind limb. Data are mean±SEM. *P<0.01 vs control group; †P<0.05 vs naked AM group.
AM-gelatin groups compared with that in the control group (Figure 3, A through C). Quantitative analysis of collateral vessels demonstrated that the angiographic score in both the naked AM and AM-gelatin groups was significantly higher than that in the control group (Figure 3D). Angiographic score did not significantly differ between the control group and Lac Z-gelatin group.

To examine the development of collateral vessels in an earlier stage, other rabbits (n=4 each) were examined 2 weeks after gene transfer (day 24). Angiograms showed significant collateral development in the naked AM and AM-gelatin groups compared with that in the control group.

**Histological Examination**

Alkaline phosphatase staining of ischemic hind limb muscle showed marked augmentation of neovascularization in both the naked AM and AM-gelatin groups compared with the control group (Figure 4, A through C). Quantitative analysis demonstrated that capillary density of the ischemic adductor muscle was highest in the AM-gelatin group (Figure 4D). Analysis of the capillary/muscle fiber ratio yielded similar results. Seven days after gene transfer, intense immunostaining for Ki67 was observed in vascular endothelial cells of the naked AM and the AM-gelatin groups (Figure 4, E through G).

**AM Expression and Akt Phosphorylation After Gene Transfer**

Seven days after gene transfer, modest immunostaining for human AM was observed in the naked AM group, whereas AM immunoreactivity was intense surrounding the gelatin in the AM-gelatin group (Figure 5, A through C). Tissue content of human AM was significantly increased both in the naked AM and the AM-gelatin groups 7 days after gene transfer (Figure 5D). The AM level in the AM-gelatin group was significantly higher that in the naked AM group. Two weeks after gene transfer, AM overexpression was observed only in the AM-gelatin group. The expression of endogenous VEGF and its receptors (Flt-1 and Flk-1) did not differ among the 3 groups (data not shown). Western blot analysis revealed that phosphorylated Akt in ischemic muscles was increased in both the naked AM and AM-gelatin groups 7 days after gene transfer (Figure 5E). Intense immunostaining for phosphorylated Akt in ischemic muscles was increased in both the naked AM and AM-gelatin groups 7 days after gene transfer (Figure 5E). 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PI3K/Akt pathway is considered to regulate multiple phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Thus, AM, an angiogenic factor. VEGF is known to induce angiogenesis. Nevertheless, angiographic collateral development and high capillary density were observed in ischemic muscles after AM gene transfer. Ki67, a marker for cell proliferation, was detected in endothelial cells of microvessels after AM gene transfer. These results suggest that AM overproduction resulting from gene transfer may induce angiogenesis in a rabbit model of hind limb ischemia. Recent studies using AM gene knockout mice have shown that AM is essential for development of the vasculature during embryogenesis. These studies support our results that AM may be an angiogenic factor. VEGF is known to induce angiogenesis and to regulate endothelial cell survival through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Thus, the PI3K/Akt pathway is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation. A recent study has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells at least in part through the PI3K/Akt pathway. The present study demonstrated that phosphorylated Akt is increased at least in endothelial cells after AM gene transfer. AM gene transfer did not influence endogenous VEGF and its receptors. Taken together, it is interesting to speculate that AM may directly induce angiogenesis through the PI3K/Akt pathway.

In the present study, we used positively charged biodegradable gelatin as a nonviral vector. We have shown that basic fibroblast growth factor (bFGF) is ionically linked with gelatin, which enhances the angiogenic effects of bFGF by delaying protein degradation. Thus, biodegradable gelatin has been used as a carrier of protein. However, little information is available regarding the therapeutic potential of gelatin as a nonviral vector for gene transfer. In the present study, we demonstrated that RITC-labeled AM DNA was incorporated into positively charged gelatin. In addition, intramuscular administration of AM DNA-gelatin complexes strongly enhanced AM production compared with that of naked AM DNA. These results suggest that biodegradable gelatin may serve as a vector for gene transfer. In fact, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hind limb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, laser Doppler flow, and muscle weight ratio and a decrease in necrosis of lower hind limb and thigh muscles. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. The angiogenic effects of AM-gelatin complexes were comparable to those of bFGF-gelatin complexes (data not shown). AM DNA-gelatin complexes were distributed mainly in connective tissues. We have recently demonstrated that gelatin-DNA complex is readily phagocytosed by mac-

Figure 5. A through C, Immunohistochemistry for human AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification ×200. D, Time course of AM production in ischemic muscles after gene transfer. Data are mean ± SEM. *P < 0.01 vs control group; †P < 0.01 vs naked AM group. E, Western blot analysis for Akt phosphorylation in muscles. F, Immunohistochemical staining for phosphorylated Akt 7 days after gene transfer. Phosphorylated Akt was distributed at least in endothelial cells. Magnification ×400.
rophages, monocytes, endothelial progenitor cells, and so on, resulting in gene expression within these phagocytes.23,24 These findings raise the possibility that AM secreted from these cells acts on muscles in a paracrine fashion. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 weeks. Thus, it is interesting to speculate that delaying gene degradation by gelatin may be responsible for the highly efficient gene transfer.

Currently, a highly efficient and safe gene delivery system is needed for gene therapy in humans. The present study demonstrated that the use of gelatin, which is considered to be less biohazardous than viral vectors, enhanced the angiogenic potential of AM DNA. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular diseases. However, the initial success of gelatin-mediated AM gene therapy reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

**Study Limitation**

First, histological capillary density, calf blood pressure ratio, and laser Doppler flow were significantly higher in the AM-gelatin group than in the naked AM group. However, the angiographic score did not significantly differ between the two. This discrepancy raises the possibility that conventional angiography may have insufficient resolution to fully visualize the angiogenic microvessels. Second, human AM level was slightly elevated in the control group. This implies that the anti-human AM antibody used in this radioimmunoassay had some cross-reactivity with endogenous rabbit AM. Nevertheless, human AM level in the muscles was highest in the AM-gelatin group within 2 weeks after gene transfer. These results suggest that AM DNA-gelatin complexes induces potent and long-lasting AM production.

**Conclusions**

Intramuscular administration of AM DNA induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases.

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