endothelial progenitor cells expanded ex vivo, *Nat. Med.*, 7: 1035-1040, 2001

22. Crosby J.R., Kaminski W.E., Schatteman G., Martin P.J., Raines E.W., Seifert R.A., Bowen Pope D.F., Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation, *Circ. Res.*, 87: 728-730, 2000

23. Murayama T., Tepper O.M., Silver M., Ma H., Losordo D.W., Isner J.M., Asahara T., Kalka C., Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo, *Exp. Hematol.*, 30: 967-972, 2002

24. Reyes M., Dudek A., Jahagirdar B., Koodie L., Marker P.H., Verfaillie C.M., Origin of endothelial progenitors in human postnatal bone marrow, *J. Clin. Invest.*, 109: 337-346, 2002

25. Tamaki T., Akatsuka A., Ando K., Nakamura Y., Matsuzawa H., Hotta T., Roy R.R., Edgerton V.R., Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle, *J. Cell. Biol.*, 157: 571-577, 2002

26. Vasa M., Fichtlscherer S., Aicher A., Adler K., Urbich C., Martin H., Zeiher A.M., Dimmeler S., Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease, *Circ. Res.*, 89: E1-E7, 2001

27. Tepper O.M., Galiano R.D., Capla J.M., Kalka C., Gagne P.J., Jacobowitz G.R., Levine J.P., Gurtner G.C., Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures, *Circulation*, 106: 2781-6, 2002

28. Valgimigli M., Rigolin G.M., Fucili A., Della Porta M., Soukhomovskaia O., Malagutti P., Bugli A.M., Bragotti L.Z., Francolini G., Mauro E., Castoldi G., Ferrari R., CD34+ and Endothelial Progenitor Cells in Patients With Various Degrees of Congestive Heart Failure, *Circulation*, 110: 1209-1212, 2004

29. Kalka C., Masuda H., Takahashi T., Gordon R., Tepper O., Gravereaux E., Pieczek A., Iwaguro H., Hayashi SI., Isner JM., Asahara T., Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects, *Circ. Res.*, 86: 1198-1202, 2000

30. Hattori K., Dias S., Heissig B., Hackett NR., Lyden D., Tateno M., Hicklin DJ., Zhu Z., Witte L., Crystal R.G., Moore M.A., Rafii S., Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells, J. Exp. Med., 193: 1005-1014, 2001

31. Yamaguchi J., Kusano K.F., Masuo O., Kawamoto A., Silver M., Murasawa S., Bosch Marce M., Masuda H., Losordo D.W., Isner J.M., Asahara T., Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization, *Circulation*, 107: 1322-1328, 2003

32. Heeschen C., Aicher A., Lehmann R., Fichtlscherer S., Vasa M., Urbich C., Mildner Rihm C., Martin H.,

Zeiher A.M., Dimmeler S., Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization, *Blood*, 102: 1340-1346. 2003

33. Iwakura A., Luedemann C., Shastry S., Hanley A., Kearney M., Aikawa R., Isner J.M., Asahara T., Losordo D.W., Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury, *Circulation*, 108: 3115-3121, 2003

34. Strehlow K., Werner N., Berweiler J., Link A., Dirnagl U., Priller J., Laufs K., Ghaeni L., Milosevic M., Bohm M., Nickenig G., Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation, *Circulation*, 107: 3059-3065, 2003

35. Kureishi Y., Luo Z., Shiojima I., Bialik A., Fulton D., Lefer D.J., Sessa W.C., Walsh K., The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals, *Nat. Med.*, 6: 1004-1010, 2000

36. Llevadot J., Murasawa S., Kureishi Y., Uchida S., Masuda H., Kawamoto A., Walsh K., Isner J.M., Asahara T., HMG-CoA reductase inhibitor mobilizes bone marrow—derived endothelial progenitor cells, *J. Clin. Invest.*, 108: 399-405, 2001

37. Dimmeler S., Aicher A., Vasa M., Mildner Rihm C., Adler K., Tiemann M., Rutten H., Fichtlscherer S., Martin H., Zeiher AM., HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway, *J. Clin. Invest.*, 108: 391-397, 2001

38. Lyden D., Hattori K., Dias S., Costa C., Blaikie P., Butros L., Chadburn A., Heissig B., Marks W., Witte L., Wu Y., Hicklin D., Zhu Z., Hackett N.R., Crystal RG., Moore M.A., Hajjar K.A., Manova K., Benezra R., Rafii S., Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth, *Nat. Med.*, 7: 1194-1201, 2001

39. Kocher A.A., Schuster M.D., Szabolcs M.J., Takuma S., Burkhoff D., Wang J., Homma S., Edwards N.M., Itescu S., Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function, *Nat. Med.*, 7: 430-436, 2001

40. Taguchi A., Soma T., Tanaka H., Kanda T., Nishimura H., Yoshikawa H., Tsukamoto Y., Iso H., Fujimori Y., Stern D.M., Naritomi H., Matsuyama T., Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model, *J. Clin. Invest.*, 114: 330-338, 2004

41. Nagaya N., Kangawa K., Kanda M., Uematsu M., Horio T., Fukuyama N., Hino J., Harada Shiba M., Okumura H., Tabata Y., Mochizuki N., Chiba Y., Nishioka K., Miyatake K., Asahara T., Hara H., Mori H., Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells, *Circulation*, 108: 889-895, 2003

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Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats

Takafumi Fujii,[1] Noritoshi Nagaya,[2,3] Takashi Iwase,[2] Shinsuke Murakami,[2]
Yoshinori Miyahara,[1] Kazuhiro Nishigami,[3] Hatsue Ishibashi-Ueda,[5] Mikiyasu Shirai,[1]
Takefumi Itoh,[2] Kozo Ishino,[6] Shunji Sano,[6] Kenji Kangawa,[4] and Hidezo Mori[1]

Departments of [1]Cardiac Physiology, [2]Regenerative Medicine and Tissue Engineering, [3]Internal Medicine,
[4]Biochemistry, and [5]Pathology, National Cardiovascular Center, Osaka; and [6]Department
of Cardiovascular Surgery, Okayama University Medical School, Okayama, Japan

Fujii, Takafumi, Noritoshi Nagaya, Takashi Iwase, Shinsuke Murakami, Yoshinori Miyahara, Kazuhiro Nishigami, Hatsue Ishibashi-Ueda, Mikiyasu Shirai, Takefumi Itoh, Kozo Ishino, Shunji Sano, Kenji Kangawa, and Hidezo Mori. Adrenomedullin enhances therapeutic potency of bone marrow transplantation for myocardial infarction in rats. Am J Physiol Hecr𝑡Circ Physiol 288: H1444–H1450, 2005. First published November 11, 2004; doi: 10.1152/ajpheart.00266.2004.—Adrenomedullin (AM), a potent vasodilator, induces angiogenesis and inhibits cell apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. Transplantation of bone marrow-derived mononuclear cells (MNC) induces angiogenesis. We investigated whether infusion of AM enhances the therapeutic potency of MNC transplantation in a rat model of myocardial infarction. Immediately after coronary ligation, bone marrow-derived MNC (5 × 10^6 cells) were injected into the ischemic myocardium, followed by subcutaneous administration of 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ AM (AM-MNC group) or saline (MNC group) for 3 days. Another two groups of rats received subcutaneous administration of AM alone (AM group) or saline (control group). Hemodynamic and histological analyses were performed 4 wk after treatment. Cardiac infarct size was significantly smaller in the MNC and AM groups than in the control group. A combination of AM infusion and MNC transplantation demonstrated a further decrease in infarct size. Left ventricular (LV) maximum change in pressure over time and LV fractional shortening were significantly improved only in the AM-MNC group. AM significantly increased capillary density in ischemic myocardium, suggesting the angiogenic potency of AM. AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Although MNC apoptosis was frequently observed 72 h after transplantation, AM markedly decreased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells among the transplanted MNC. In conclusion, AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC.

angiogenesis; apoptosis; mononuclear cell

DESPITE THE RECENT REMARKABLE progress in medical and surgical treatment for ischemic heart disease, this disease remains a major cause of death worldwide (5). Bone marrow-derived mononuclear cells (MNC) contain various kinds of cell lineages and numerous cytokines that contribute to neovascularization (1, 15). In fact, autologous transplantation of bone marrow cells has been shown to enhance angiogenesis and improve cardiac function in an animal model of cardiac ischemia (6, 9, 10). Recent human studies have demonstrated beneficial effects of transplanted MNC in patients with ischemic heart disease (23, 25). However, some patients fail to respond to this cell therapy. Thus a novel therapeutic strategy to enhance the angiogenic property of MNC is desirable.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (8). We have shown that infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure (17). On the other hand, AM has been shown to activate the phosphatidylinositol 3-kinase (PI3-kinase)/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis including endothelial cell proliferation, migration, and capillary-like formation (14, 22). In fact, we have shown that AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia via activation of Akt (24). These findings suggest that AM may play an important role in the regulation of vascular regeneration. In addition, AM has been shown to exert an antiapoptotic effect on a variety of cells including vascular endothelial cells (7, 20). Taking these findings together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of ischemic heart disease.

Thus the purposes of this study were 1) to investigate whether infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of myocardial infarction, and 2) to investigate the effects of AM on survival and differentiation of the transplanted MNC to examine the underlying mechanisms of the effects induced by AM.

MATERIALS AND METHODS

Animal model. Myocardial infarction was produced in male Lewis rats weighing 200–220 g by left coronary ligation. In brief, after rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt), they were ventilated artificially. The heart was exposed via left thoracotomy, and the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium using a 6-0 proline suture. Finally, the heart was restored to its normal position, and the chest was closed. The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

Address for reprint requests and other correspondence: N. Nagaya, Dept. of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan (E-mail: nnagaya@ri.ncvc.go.jp).

Preparation of MNC. After Lewis rats were killed, bone marrow from the femur and tibia was collected and put in PBS. Marrow cells were loaded on a 1.077 gradient of Ficoll (Lymphoprep; Nycomed Pharma, Oslo, Norway) and centrifuged at 1,500 rpm for 20 min. The cells were then washed with 10 ml PBS to remove the Ficoll and centrifuged at 2,000 rpm for 10 min. The cells were finally suspended in PBS at a concentration of 5×10^6 cells in 50 µl PBS for transplantation. Fluorescence-activated cell sorting analysis demonstrated that 22 ± 1% of MNC were positive for lectin from ulex europaeus (UEA)-1 lectin (Sigma, St. Louis, MO).

MNC transplantation and AM infusion. Transplantation of bone marrow-derived MNC and/or 3-day infusion of AM was performed immediately after coronary ligation. MNC (5×10^6 cells in 50 µl PBS) were injected into the myocardium at five points in the border zone surrounding the infarct by using a 27-gauge needle. Recombinant human AM (0.05 µg·kg^{-1}·min^{-1}) was subcutaneously administered by using an osmotic minipump (model 2004; Alza, Palo Alto, CA) for 3 days. The pump was positioned in a pocket constructed in the subcutaneous tissue just below the subscapular region. For control, 5% glucose was infused in a similar manner in the rats receiving coronary ligation. This protocol resulted in the creation of four groups: *1*) AM infusion plus MNC transplantation (AM-MNC group, $n = 15$), *2*) vehicle infusion plus MNC transplantation (MNC group, $n = 14$), *3*) AM infusion plus PBS injection (AM group, $n = 14$), and *4*) vehicle infusion plus PBS injection (control group, $n = 13$).

Echocardiographic studies. Echocardiographic studies were performed 4 wk after surgery using a 7.5-MHz phased-array transducer (model HP SONOS 5500; Hewlett-Packard, Andover, MA). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt) as a supplement to maintain mild anesthesia. M-mode tracings were obtained at the level of the papillary muscles. Anterior and posterior end-diastolic wall thickness, left ventricular (LV) end-diastolic and end-systolic dimension, and LV fractional shortening were measured from three consecutive cardiac cycles by the American Society for Echocardiology leading-edge method (21).

Cardiac catheterization. Cardiac catheterization was performed 4 wk after surgery. Rats were anesthetized with intraperitoneal pentobarbital and placed on a heating pad to maintain body temperature at 37–38°C throughout the study. A 1.5 Fr micronanometer-tipped catheter was inserted in the right carotid artery for measurement of heart rate and mean arterial pressure. The catheter was then advanced into the LV for measurement of LV end-diastolic pressure and then replaced with a thermomicroprobe for measurements of cardiac output. These hemodynamic variables were measured with a pressure transducer (UFI, Morro Bay, CA) connected to a polygraph and recorded with a thermal recorder (model 7758 B system; Hewlett-Packard).

Infarct size measurement. After completion of hemodynamic measurements, the heart was arrested by an injection of 2 mmol KCl through the carotid artery, and the cardiac ventricles were excised. The size of myocardial infarction was determined by a previously described method (2). In brief, incisions were made in the LV so that the tissue could be pressed flat. The circumference of the entire flat LV and the visualized infarcted area, as judged from both the epicardial and endocardial sides, was outlined on a clear plastic sheet. The difference in weight between the two marked areas on the sheet was used to determine infarction size and was expressed as a percentage of LV surface area.

Histological analysis of microvessel density. LV myocardium was fixed in 10% formalin. Three cross sections of the LV, cut from apex to base, were obtained from individual rats for comparison among four groups ($n = 5$ each). They were embedded in paraffin and stained with Masson's trichrome for measurement of interstitial fibrosis. In other rats ($n = 5$ each), LV myocardium was embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in liquid nitrogen, and cut into 5-µm-thick sections. Tissue sections were stained for alkaline phosphatase with an indoxyltetrazolium method to detect capillary endothelial cells ($n = 5$ in each group). The number of capillary vessels was counted in the peri-infarct area (a 1.0-mm band next to the scar) excluding scar region using a light microscope at a magnification of ×200. The numbers in five high-power fields in each rat were averaged and expressed as the number of capillary vessels. These morphometric studies were performed by two examiners who were blinded to treatment.

Detection of MNC apoptosis. To examine the antiapoptotic effect of AM on transplanted MNC, red fluorescence-labeled MNC were transplanted into ischemic myocardium in rats with ($n = 5$) and without ($n = 5$) AM infusion. Before implantation into the ischemic heart, suspended MNC were labeled with fluorescent dyes with a PKH26 (Red Fluorescent Cell Linker Kit; Sigma), as reported previously (13). AM was subcutaneously administered by using a minipump for 3 days. Rats were killed 72 h after MNC transplantation. The LV was enucleated, and muscle samples were embedded in OCT compound and snap frozen in liquid nitrogen for the detection of apoptosis. Serial sections of the heart were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) for apoptosis using an in situ apoptosis detection kit (model S7111 Apoptag Fluorescein Kit; Intergen). Apoptosis of transplanted MNC was also evaluated by the detection of cleaved caspase-3-positive cells. In brief, the frozen tissue sections were incubated with anticleaved caspase-3 antibody (Cell Signaling), followed by incubation with FITC-conjugated IgG antibody (BD Pharmingen, San Diego, CA). The number of TUNEL/PKH26 double-positive cells and caspase-3/PKH26 double-positive cells was counted in 10 fields of each rat using a confocal microscopy (Fluoview model 500; Olympus, Tokyo, Japan).

The antiapoptotic effect of AM on MNC was also evaluated by in vitro TUNEL assay. MNC were plated on 12-well plates (1×10^6 cells per well) and cultured in serum-free medium for 24 h with control buffer, AM (1×10^{-7} M), or AM plus wortmannin, a PI3-kinase inhibitor (50 nM). Randomly selected microscopic fields ($n = 10$) were evaluated for calculating the ratio of TUNEL-positive cells to total cells.

Monitoring of implanted MNC in ischemic heart. Additional rats were used to examine whether transplanted MNC differentiate into endothelial cells, cardiomyocytes, vascular smooth muscle cells, or macrophages in the ischemic heart. PKH26 (red fluorescence)-labeled MNC were injected into the ischemic heart in rats with ($n = 8$) and without ($n = 8$) AM infusion. These subgroups of rats were killed 4 wk after coronary ligation. To identify vascular endothelial cells in vivo, FITC-labeled UEA-1 lectin was intravenously administered 30 min before the rats were killed ($n = 5$ in each group). The LV was enucleated, and muscle samples were then embedded in OCT compound, snap frozen in liquid nitrogen, and cut into sections. Sections were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) to detect nuclei. The number of DAPI/PKH26 double-positive cells and lectin-positive cells in the peri-infarct area was counted in 10 fields of each rat using a confocal microscopy. Frozen sections from other rats ($n = 3$ in each group) were incubated with mouse anticardiac troponin T (Novocastra, Newcastle, UK), anti-α-smooth muscle actin antibody (Dako, Copenhagen, Denmark), and anti-ED1 antibody (Serotec, Oxford, UK), followed by incubation with FITC-conjugated IgG antibody. In other rats (MNC group, $n = 5$; AM-MNC group, $n = 5$), the cardiac muscle from base to apex was transversely cut into 6-µm slices to calculate the number of transplanted MNC present within the heart 4 wk after transplantation. These morphometric studies were performed by two examiners who were blinded to treatment.

Statistical analysis. Numerical values were expressed as means ± SE. Comparisons of parameters among the four groups were performed by one-way ANOVA, followed by Newman-Keuls test for unpaired data. Comparisons of parameters between two groups were made by unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.
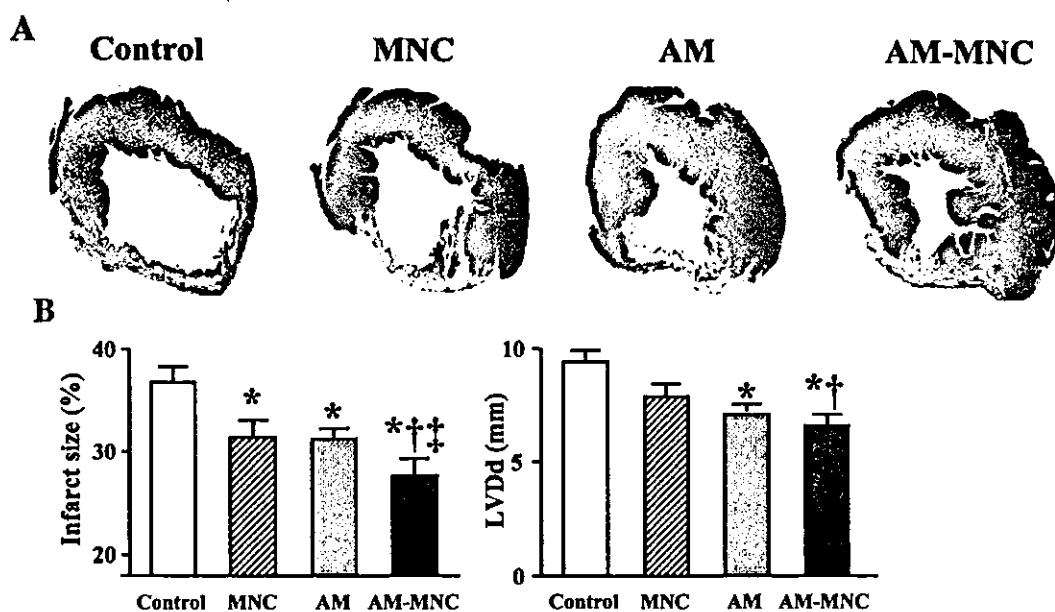
Fig. 1. A: representative examples of Masson trichrome-staining of transverse sections of left ventricular (LV) myocardium 4 wk after coronary ligation. B: quantitative analysis of infarct size and LV chamber size. Infarcted area and LV end-diastolic diameter (LVDd) of the adrenomedullin-mononuclear cell (AM-MNC) group were significantly smaller than those of the other groups. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

RESULTS

Infarct size and ventricular weight. Moderate-to-large infarcts were observed in the control group after coronary ligation (Fig. 1). However, infarct size was smaller in the MNC, AM, and AM-MNC groups than in the control group. In particular, it was very small in the AM-MNC group. Quantitative analysis also demonstrated that cardiac infarct size in the AM-MNC group was smallest among the four groups. Right ventricular weight was significantly lower in the AM and AM-MNC groups than that in the control group (Table 1). LV weight did not significantly differ among the four groups.

Echocardiographic findings. LV diastolic dimension was smallest in the AM-MNC group, followed by the AM, MNC, and control groups (Fig. 1). LV fractional shortening in the AM-MNC group was also higher than that in the control, MNC, and AM groups (Table 2). Diastolic thickness of the anterior wall was significantly attenuated in the MNC, AM, and AM-MNC groups compared with the control group.

Hemodynamics. Cardiac output in the AM-MNC group was significantly higher than that in the control, MNC, and AM groups (Fig. 2). LV end-diastolic pressure in the MNC, AM, and AM-MNC groups was significantly lower than that in the control group. LV maximum change in pressure over time (dP/dt) in the MNC and AM-MNC group were significantly higher than that in the control group. Similarly, LV minimum dP/dt was significantly decreased only in the AM-MNC group.

Capillary density. Alkaline phosphatase staining of ischemic myocardium showed marked augmentation of neovascularization in the MNC, AM, and AM-MNC groups compared with the control group (Fig. 3A). Quantitative analysis demonstrated that capillary density was significantly higher in the AM-MNC group than in the MNC and AM groups (Fig. 3B). Cartilage, bone, or fat was not observed in the transplanted area. No tumor-like cells were seen.

Antiapoptotic effect of AM on MNC. Red fluorescence-labeled MNC were detected in each recipient heart 72 h after transplantation (Fig. 4). TUNEL-positive cells were frequently observed in the MNC group. In contrast, these apoptotic cells

Table 1. *Physiological profiles of four experimental groups*

	Control	MNC	AM	AM-MNC
Number	13	14	14	15
Body weight, g	274±3	285±5	287±3	305±4*
Heart rate, bpm	410±24	404±30	398±33	387±36
MAP, mmHg	101±11	104±13	103±9	116±14*
LV wt/body wt, g/kg	2.4±0.2	2.5±0.2	2.6±0.1	2.5±0.2
RV wt/body wt, g/kg	1.1±0.1	0.9±0.1	0.8±0.1*	0.7±0.1*

Values are means ± SE; number is number of rats in each group. Control group, myocardial infarction rats given vehicle; MNC group, those given mononuclear cells; AM, those given adrenomedullin; AM-MNC, those given AM and MNC; MAP, mean arterial pressure; LV, left ventricle; RV, right ventricle. *$P < 0.05$ vs. control.

Table 2. *Echocardiographic findings*

	Control	MNC	AM	AM-MNC
LVDd, mm	9.9±0.2	8.3±0.3	7.3±0.2*	6.9±0.3*†
LVDs, mm	8.4±0.3	6.6±0.4	5.8±0.2*	5.1±0.2*
%FS, %	14±1	22±1*	21±1*	26±1*†‡
AWT diastole, mm	1.0±0.2	1.3±0.3*	1.3±0.3*	1.4±0.4*
PWT diastole, mm	1.5±0.5	2.2±0.4	2.1±0.4	2.2±0.4

Values are means ± SE. LVDd, LV diastolic dimension; LVDs, LV systolic dimension; %FS, LV fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.
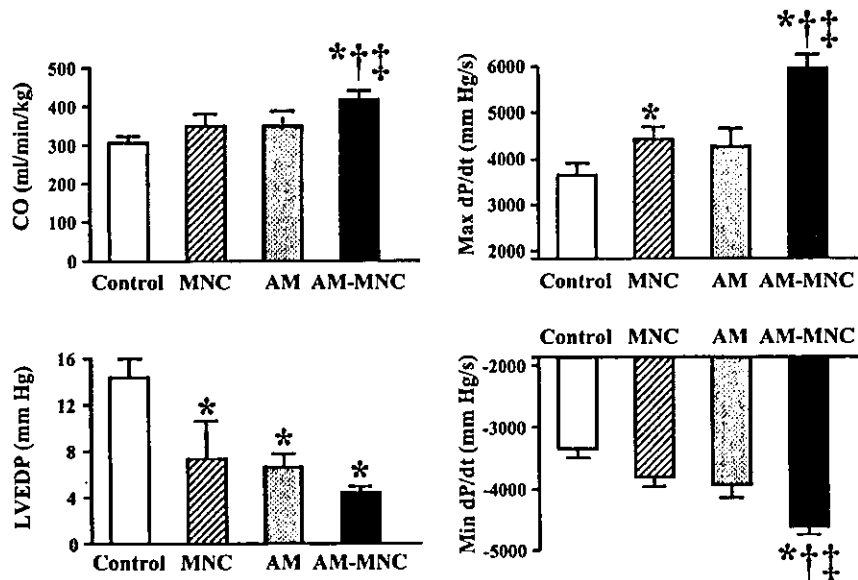
Fig. 2. Effects of AM infusion and MNC transplantation on hemodynamic parameters. CO, cardiac output; LVEDP, LV end-diastolic pressure; Max dP/dt, LV maximum change in pressure over time; Min dP/dt, LV minimum dP/dt. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

were hardly detected in the AM-MNC group. Semiquantitative analysis demonstrated that the number of TUNEL-positive MNC was significantly lower in the AM-MNC group than in the MNC group. Similarly, the number of caspase-3-positive MNC was significantly lower in the AM-MNC group than in the MNC group. These results suggest that infusion of AM inhibits apoptosis of transplanted MNC.

In vitro, serum starvation induced MNC apoptosis. When incubated in the presence of AM (1×10^{-7} M), the percentage of TUNEL-positive cells decreased significantly (19 ± 1 to $9 \pm 1\%$, $P < 0.05$). However, pretreatment with wortmannin, a PI3-kinase inhibitor, diminished the antiapoptotic effect of AM ($17 \pm 1\%$).

Differentiation of MNC into endothelial lineage. Four weeks after transplantation, fluorescence-labeled transplanted cells
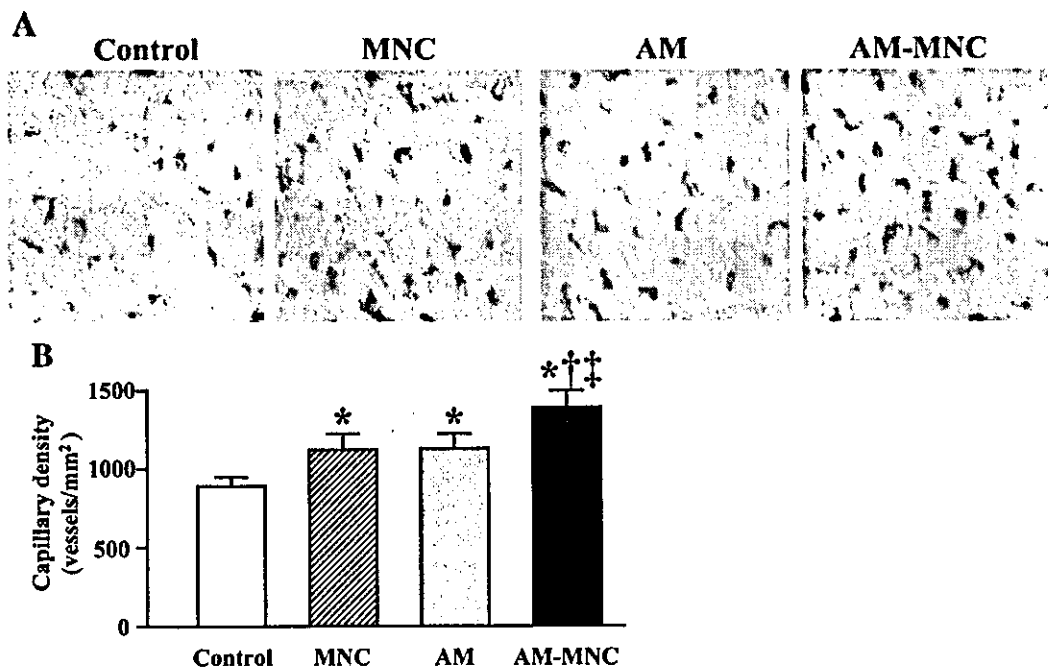

Fig. 3. *A*: representative examples of alkaline phosphatase staining in peri-infarct area. A combination of AM infusion and MNC transplantation markedly induced myocardial neovascularization. Magnification, ×200. *B*: quantitative analysis of capillary density in peri-infarct area. Capillary density in the AM-MNC group was significantly higher than that in the MNC and AM groups. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.
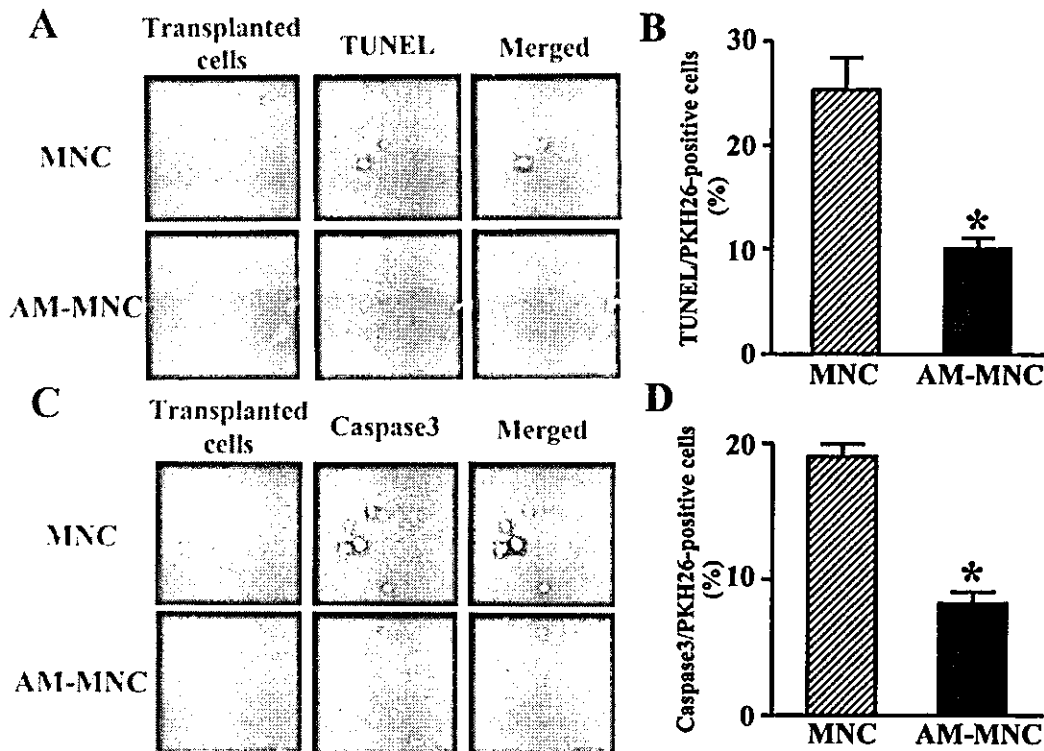
Fig. 4. Detection of transplanted cell apoptosis. *A*: representative photographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates TUNEL-positive cells. TUNEL-positive cells were frequently observed in the MNC group, whereas they were hardly detected in the AM-MNC group. Magnification, ×400. *B*: semiquantitative analysis of TUNEL-positive cells in the PKH26-positive (transplanted) cells. *C*: representative photographs of caspase-3 staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates caspase-3-positive cells. *D*: semiquantitative analysis of caspase-3-positive cells in the PKH26-positive cells. Values are means ± SE. *P < 0.05 vs. control.

were more frequently observed in the AM-MNC group than in the MNC group (6.4 ± 0.4 to 3.1 ± 0.2%, $P < 0.05$). Moreover, some of the transplanted cells were positive for UEA-1 lectin in the AM-MNC group (Fig. 5A), suggesting differentiation of MNC into vascular endothelial cells. Semiquantitative analysis demonstrated that the number of DAPI/PKH26 double-positive cells (viable transplanted cells) was significantly higher in the AM-MNC group than in the MNC group (Fig. 5B). Moreover, the ratio of lectin-positive cells to DAPI/PKH26 double-positive cells was significantly higher in the AM-MNC group than in the MNC group. The ratio of DAPI/PKH26 double-positive cells to lectin-positive cells was small, but significantly higher in the AM-MNC group than in the MNC group (23.9 ± 0.9 to 17.2 ± 0.6%, $P < 0.01$). Transplanted MNC were negative for troponin T or α-smooth muscle actin-positive cells. Some of the transplanted MNC were positive for ED1, a marker of macrophage (data not shown).

DISCUSSION

In the present study, we demonstrated that *1*) infusion of AM enhanced the angiogenic potency of MNC in a rat model of acute myocardial infarction, resulting in decreased infarct size and improved cardiac function. We also demonstrated that *2*) AM induced angiogenesis and inhibited apoptosis of the transplanted MNC. Thus a combination of AM and MNC may have beneficial effects in rats with myocardial infarction, partly

through the angiogenic potency of AM itself and through its antiapoptotic effect on MNC.

Bone marrow-derived MNC include a variety of stem and progenitor cells (1, 15, 19), some of which can differentiate into endothelial cells and secrete numerous cytokines and chemokines (6, 9, 10). Earlier studies (6, 9, 10, 23, 25) have shown that autologous bone marrow transplantation induces angiogenesis and improves LV function in animals and humans. However, some patients are refractory to this cell therapy. Thus an approach to augment the angiogenic potency of MNC transplantation is required.

The present study showed that MNC transplantation or AM infusion alone reduced infarct size. A combination of AM infusion and MNC transplantation resulted in further decreases in infarct size and LV chamber size. MNC transplantation or AM administration modestly improved LV function. On the other hand, a combination of MNC and AM significantly improved cardiac performance compared with MNC or AM alone, as indicated by increases in cardiac output, fractional shortening, and LV maximum dP/dt. Earlier studies (6, 9, 10) have reported that MNC transplantation induces therapeutic angiogenesis and preserves LV function through inhibition of cardiomyocyte apoptosis in animal models of myocardial infarction. We have shown that AM infusion during the acute phase of ischemia-reperfusion inhibits apoptosis of cardiomyocytes and produces hemodynamic improvement in an animal
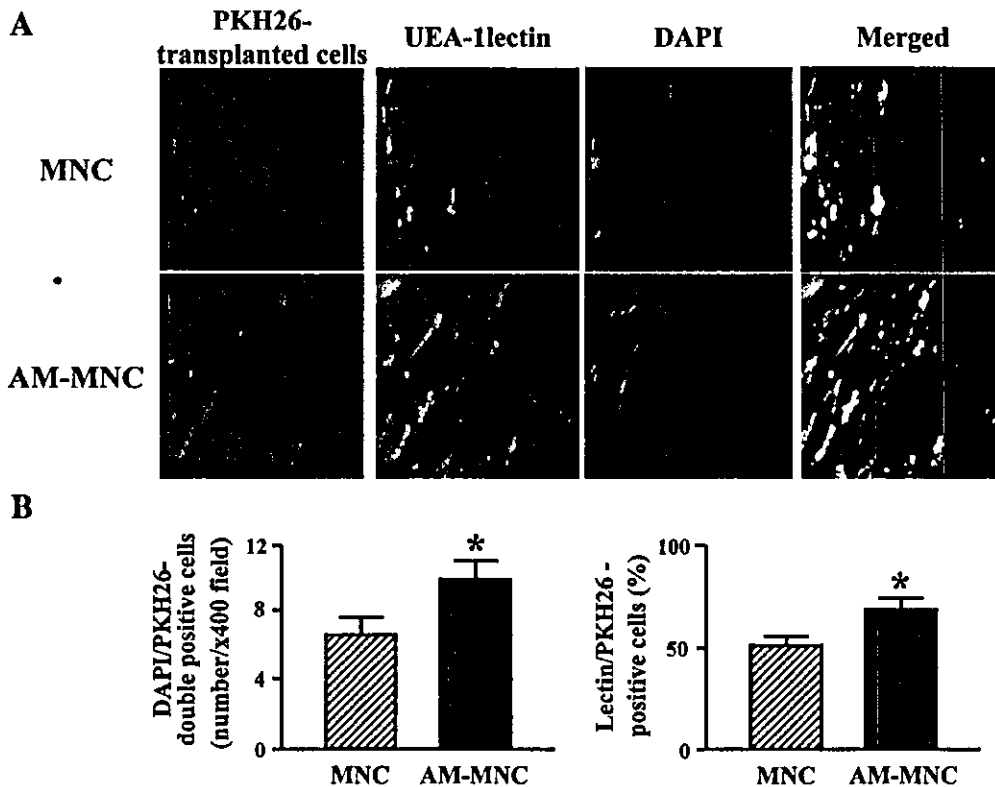
Fig. 5. A: representative examples of MNC differentiation into endothelial lineage. Red fluorescence (PKH26) marks transplanted cells; green fluorescence indicates ulex europaeus (UEA)-1 lectin, a marker for vascular endothelial cells. Most of the transplanted cells differentiated into endothelial cells in the AM-MNC group. Magnification, ×400. B: quantitative analysis of living transplanted cells and endothelial differentiation. The number of living cells after transplantation was significantly higher in the AM-MNC group than in the MNC group. The ratio of lectin-positive cells to living transplanted cells was significantly higher in the AM-MNC group than in the MNC group. Values are means ± SE. *P < 0.05 vs. control. DAPI, 4',6'-diamidino-2-phenylindole.

study (18). These findings suggest that the reduction of infarct size induced by this combination therapy may be attributable to additive cardioprotective effects of MNC and AM.

The present study showed that AM infusion significantly increased capillary density in ischemic myocardium. Furthermore, AM infusion plus MNC transplantation demonstrated a further increase in capillary density compared with AM or MNC alone. Contribution of transplanted MNC to neovascularization (the ratio of DAPI/PKH26 double-positive cells to lectin-positive cells) was significantly greater in the AM-MNC group than in the MNC group. A recent study (14) has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells and enhances angiogenesis in a murine gel plug assay through the PI3-kinase/Akt pathway. We have also shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a rabbit model of chronic hindlimb ischemia via activation of Akt (24). These findings suggest that the beneficial effects of combination therapy using AM and MNC may be attributable, in part, to the angiogenic properties of AM itself. Thus it is possible that AM infusion and MNC transplantation induce additive effects on myocardial damage after myocardial infarction. However, it still remains unknown whether AM infusion plus MNC transplantation induces synergetic effects.

An earlier study has demonstrated that ischemia and mechanical stress induce apoptosis of transplanted cells in the early stage after MNC transplantation (9). These results raise the possibility that the angiogenic potency of MNC transplantation is attenuated by MNC apoptosis. Kim et al. (7) have demonstrated that AM inhibits apoptosis of endothelial cells through the PI3-kinase/Akt pathway in vitro. Activation of the PI3-kinase/Akt pathway has been shown to inhibit apoptosis of endothelial progenitor cells and enhance neovascularization (11). In the present study, AM infusion significantly inhibited MNC apoptosis in ischemic tissue. In vitro, we showed that the antiapoptotic effect of AM on MNC was mediated by activation of the PI3-kinase/Akt pathway. Thus AM may enhance the therapeutic potency of MNC transplantation through a direct action of AM on MNC survival. Moreover, immunohistological examination demonstrated that infusion of AM increased the number of lectin-positive (endothelial) cells in transplanted MNC. These findings raise the possibility that AM may enhance differentiation of MNC into the endothelial lineage. Thus AM may directly act on transplanted MNC, which may result in synergetic effects on the ischemic myocardium.

This study includes some study limitations. Although the labeling efficacy of PKH26 has been shown to persist for >8 wk without cell toxicity (3, 4), the used vital marker PKH26

may have some cell toxic effects and cell or membrane fusion can lead to labeling of neighboring cells in the target tissue. Second, the present study demonstrated that AM prolongs MNC survival through the PI3-kinase/Akt pathway and enhances neovascularization in a peri-infarcted area. However, further studies are necessary to examine the effect of AM on MNC differentiation into endothelial cells.

Autologous cell transplantation may be an alternative treatment for ischemic heart disease in the clinical setting. Because their use does not require immunosuppression, the clinical use of MNC for cellular cardiomyoplasty appears to be most advantageous. Administration of AM peptide is simple and relatively noninvasive. We and others (12, 16, 17) have reported the safety of AM infusion in humans. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

In conclusion, infusion of AM enhanced the angiogenic potency of MNC transplantation and improved cardiac function in rats with myocardial infarction. This beneficial effect may be mediated partly by the angiogenic property of AM itself and by its antiapoptotic effect on MNC. Thus combination therapy using AM infusion and MNC transplantation may be a new therapeutic strategy for the treatment of ischemic heart disease.

GRANTS

REFERENCES

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, and Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275: 964–967, 1997.
2. Chien YW, Barbee RW, MacPhee AA, Frohlich ED, and Trippodo NC. Increased ANF secretion after volume expansion is preserved in rats with heart failure. *Am J Physiol Regul Integr Comp Physiol* 254: R185–R191, 1988.
3. Fox D, Kouris GJ, Blumofe KA, Heilizer TJ, Husak V, and Greisler HP. Optimizing fluorescent labeling of endothelial cells for tracking during long-term studies of autologous transplantation. *J Surg Res* 86: 9–16, 1999.
4. Gulbins H, Pritisanac A, Anderson I, Uhlig A, Goldemund A, Dae-britz S, Meiser B, and Reichart B. Myoblasts for survive 16 weeks after intracardiac transfer and start differentiation. *Thorac Cardiovasc Surg* 51: 295–300, 2003.
5. Hennekens CH. Increasing burden of cardiovascular disease: current knowledge and future directions for research on risk factors. *Circulation* 97: 1095–1102, 1998.
6. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani S, Murohara T, Imaizumi T, and Iwasaka T. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 104: 1046–1052, 2001.
7. Kim W, Moon SO, Sung MJ, Kim SH, Lee S, So JN, and Park SK. Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. *FASEB J* 17: 1937–1939, 2003.
8. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, and Eto T. Adrenomedullin: a novel hypotensive peptide

isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 192: 553–560, 1993.
9. Kobayashi T, Hamano K, Li TS, Katoh T, Kobayashi S, Matsuzaki M, and Esato K. Enhancement of angiogenesis by the implantation of self bone marrow cells in a rat ischemic heart model. *J Surg Res* 89: 189–195, 2000.
10. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, and Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 7: 430–436, 2001.
11. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, and Asahara T. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J Clin Invest* 108: 399–405, 2001.
12. McGregor DO, Troughton RW, Frampton C, Lynn KL, Yandle T, Richards AM, and Nicholls MG. Hypotensive and natriuretic actions of adrenomedullin in subjects with chronic renal impairment. *Hypertension* 37: 1279–1284, 2001.
13. Messina LM, Podrazik RM, Whitehill TA, Ekhterae D, Brothers TE, Wilson JM, Burkel WE, and Stanley JC. Adhesion and incorporation of lacZ-transduced endothelial cells into the intact capillary wall in the rat. *Proc Natl Acad Sci USA* 89: 12018–12022, 1992.
14. Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi-Kobayashi T, Park K, and Nakao K. Adrenomedullin provokes endothelial Akt activation and promotes vascular regeneration both in vitro and in vivo. *FEBS Lett* 544: 86–92, 2003.
15. Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, Onitsuka I, Matsui K, and Imaizumi T. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest* 105: 1527–1536, 2000.
16. Nagaya N, Kyotani S, Uematsu M, Ueno K, Oya H, Nakanishi N, Shirai M, Mori H, Miyatake K, and Kangawa K. Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary hypertension. *Circulation* 109: 351–356, 2004.
17. Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, Oya H, Kyotani S, Nakanishi N, Goto Y, Masuda Y, Miyatake K, and Kangawa K. Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation* 101: 498–503, 2000.
18. Okumura H, Nagaya N, Itoh T, Okano I, Hino J, Mori K, Tsukamoto Y, Ishibashi-Ueda H, Miwa S, Tambara K, Toyokuni S, Yutani C, and Kangawa K. Adrenomedullin infusion attenuates myocardial ischemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway. *Circulation* 109: 242–248, 2004.
19. Rafii S and Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 9: 702–712, 2003.
20. Sata M, Kakoki M, Nagata D, Nishimatsu H, Suzuki E, Aoyagi T, Sugiura S, Kojima H, Nagano T, Kangawa K, Matsuo H, Omata M, Nagai R, and Hirata Y. Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis via a cyclic GMP-independent mechanism. *Hypertension* 36: 83–88, 2000.
21. Schiller NB, Shah PM, Crawford M, DeMaria A, Devereux R, Feigenbaum H, Gutgesell H, Reichek N, Sahn D, Schnittger I, Silverman NH, and Tajik AJ. Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. American Society of Echocardiography Committee on Standards, Subcommittee on Quantitation of Two-Dimensional Echocardiograms. *J Am Soc Echocardiogr* 2: 358–367, 1989.
22. Shiojima I and Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res* 90: 1243–1250, 2002.
23. Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, Kogler G, and Wernet P. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 106: 1913–1918, 2002.
24. Tokunaga N, Nagaya N, Shirai M, Tanaka E, Ishibashi-Ueda H, Harada-Shiba M, Kanda M, Ito T, Shimizu W, Tabata Y, Uematsu M, Nishigami K, Sano S, Kangawa K, and Mori H. Adrenomedullin gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia: benefits of a novel nonviral vector, gelatin. *Circulation* 109: 526–531, 2004.
25. Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, and Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* 361: 47–49, 2003.

ELSEVIER

Review

Adrenomedullin in the treatment of pulmonary hypertension

Noritoshi Nagaya[a,*], Kenji Kangawa[b]

[a] *Department of Internal Medicine, National Cardiovascular Center, 5-7-1 Fujisjirodai, Suita, Osaka 565-8565, Japan*
[b] *Department of Biochemistry, National Cardiovascular Center Research Institute,
5-7-1 Fujisjirodai, Suita, Osaka 565-8565, Japan*

Abstract

Adrenomedullin (AM) is a potent, long-lasting pulmonary vasodilator peptide. Plasma AM level is elevated in patients with primary pulmonary hypertension (PPH), and circulating AM is partially metabolized in the lungs. These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. We have demonstrated the effects of three types of AM delivery systems: intravenous administration, inhalation, and cell-based gene transfer. Despite endogenous production of AM, intravenously administered AM at a pharmacologic level decreased pulmonary vascular resistance in patients with PPH. Inhalation of AM improved hemodynamics with pulmonary selectivity and exercise capacity in patients with PPH. Cell-based AM gene transfer ameliorated pulmonary hypertension rats. These results suggest that additional administration of AM may be effective in patients with pulmonary hypertension. AM may be a promising endogenous peptide for the treatment of pulmonary hypertension.
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Keywords: Adrenomedullin; Pulmonary hypertension; Inhalation; Gene therapy

Contents

* Corresponding author. Tel.: +81 6 6833 5012; fax: +81 6 6833 9865.
  E-mail address: nagayann@hsp.ncvc.go.jp (N. Nagaya).

1. Introduction

Primary pulmonary hypertension (PPH) is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure, and death [42,43]. Median survival is considered to be 2.8 years from the time of diagnosis. Because the presence of endothelial injury in the pulmonary vascular bed develops pulmonary vasoconstriction, smooth muscle cell proliferation, and in situ thrombosis [1], a variety of vasodilators, anti-proliferative agents, and anticoagulants have been proposed as therapeutic agents of PPH [3,10,23,45]. Despite therapeutic medical advances including prostacyclin therapy [3,23,45], some patients ultimately require heart–lung or lung transplantation [38,41]. Thus, a novel therapeutic strategy is desirable for the treatment of pulmonary hypertension including PPH.

Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma [19]. The peptide consists of 52 amino acids with an intramolecular disulfide bond, sharing slight homology with calcitonin gene-related peptide and amylin. Immunoreactive AM has subsequently been detected in plasma and a variety of tissues including blood vessels and lungs [13,47]. AM is metabolized by neutral endopeptidase protein in the kidney and by receptor binding in a variety of tissues. The half-life of AM is approximately 15 min. Earlier studies have shown that plasma AM level is increased in patients with hypertension [14] or heart failure [34]. Taking together its potent vasodilatory effect [19] and diuretic and natriuretic effects [21], AM may be involved in the regulation of the body fluid and thus in the cardiovascular homeostasis. We have shown that plasma AM level is elevated in patients with PPH, and that the plasma AM level increases in proportion to the severity of pulmonary hypertension [16]. It has been reported that there are abundant binding sites for AM in the lungs [37]. In fact, circulating AM is partially metabolized in the lungs [52]. These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. Earlier studies have shown that AM has a variety of biological effects, which are necessary for the treatment of pulmonary hypertension (Table 1). These actions of AM are mediated by calcitonin

receptor-like receptor (CRLR) which functions as a selective AM receptor depending on the expression of the subtypes 2 and 3 of a family of receptor-activity-modifying proteins (RAMPs) [22]. AM acts through some signaling pathways: the cyclic adenosine 3′, 5′-monophosphate (cAMP), cyclic guanosine 3′, 5′-monophosphate (cGMP), phosphatidylinositol 3-kinase (PI3K)/Akt, and etc. These actions are induced by 0.01~0.1 ug/(kg min) in vivo and by 10^{-10} to 10^{-7} M in vitro. This article will summarize the therapeutic potential of AM for the treatment of pulmonary hypertension.

2. Intravenous administration of AM

In vivo studies have shown that intravenously administered AM causes vasodilation, diuresis, and a positive inotropic effect in an experimental model of heart failure [40]. In humans, intravenous administration of AM decreases systemic and pulmonary vascular resistance and increases cardiac output in patients with congestive heart failure, together with slight increases in urine volume and urinary sodium excretion [31]. Endogenous AM production is enhanced in a variety of cardiovascular diseases through a compensatory mechanism [29]. Nonetheless, additional supplementation of AM has beneficial effects in these diseases [27]. These results suggest that endogenous AM level is not sufficient enough to improve deteriorated conditions in spite of the increased AM production.

Experimental studies have shown that intralobar arterial infusion of AM causes dose-related decreases in pulmonary vascular resistance under conditions of high pulmonary vascular tone [9,20,36]. The vasodilatory effect is mediated by cAMP-dependent and nitric oxide-dependent mechanisms [15,32]. Thus, AM is known to be one of the most potent endogenous vasodilators in the pulmonary vascular bed. However, little information is available regarding the hemodynamic effects of intravenously administered AM in patients with pulmonary hypertension. Accordingly, we examined the hemodynamic and hormonal responses to intravenous infusion of AM (0.05 μg/kg/min) or placebo, were examined in 13 patients with pulmonary arterial hypertension including PPH [28]. Because AM-induced hypotension

Table 1
Beneficial effects of adrenomedullin for the treatment of pulmonary hypertension

Biological activity	Second messenger or signal
1. Potent pulmonary vasodilation	cAMP, NO/cGMP, PI3K/Akt
2. Inhibition of endothelial cell apoptosis	PI3K/Akt
3. Inhibition of smooth muscle cell proliferation and migration	cAMP, Ca^{2+}
4. Positive inotropic effect	cAMP, protein kinase C, Ca^{2+} release or influx
5. Diuresis and natriuresis	NO/cGMP, cAMP
6. Suppression of aldosterone production	Ca^{2+}
7. Induction of angiogenesis	PI3K/Akt, MEK/ERK
8. Anti-inflammation	cAMP

cAMP: cyclic adenosine 3′, 5′-monophosphate, cGMP: cyclic guanosine 3′, 5′-monophosphate, PI3K: phosphatidylinositol 3-kinase, NO: nitric oxide, ERK: extracellular signal-regulated kinase, MEK: mitogen-activated protein ERK kinase.

may cause adverse effects in patients with pulmonary hypertension, we used a relatively low dose of AM. Intravenous infusion of AM increased plasma AM level in patients with pulmonary hypertension (15 ± 1 to 48 ± 8 fmol/ml, cf. 10 ± 1 fmol/ml in healthy subjects). Infusion of AM significantly decreased pulmonary vascular resistance by 32%. In addition, AM decreased systemic vascular resistance without inducing a marked hypotension. The hemodynamic effects of AM lasted at least 15 min after the end of infusion. These results suggest that AM has potent, relatively long-lasting pulmonary vasodilator activity in patients with pulmonary hypertension. We have shown that administered AM increases plasma cAMP, but not cGMP, in patients with pulmonary hypertension, in association with its hemodynamic effects. The increase in cAMP in smooth muscle cells by AM activates protein kinase A, resulting in the decrease in calcium content in smooth muscle cells. It is therefore possible that AM may relax vascular smooth muscle through a cAMP/protein kinase A-dependent mechanism. On the other hand, Nossaman et al. [36] have shown that AM regulates pulmonary vascular tone in rats through an endothelium-derived nitric oxide-dependent mechanism. Nishimatsu et al. [35] have shown that AM induces Akt activation in the endothelium via the Ca^{2+}/calmodulin-dependent pathway and that this is implicated in the production of nitric oxide, which in turn induced endothelium-dependent vasodilation. Because the vascular effects of AM are known to vary with species and vascular regions, further studies are necessary to elucidate the mechanisms responsible for pulmonary vasodilator activity of AM in humans.

Intravenous infusion of AM markedly increased cardiac index in patients with pulmonary hypertension [28], consistent with our previous results from left sided heart failure [31]. Considering the strong vasodilator activity of AM in the systemic and pulmonary vasculature, the significant decrease in cardiac afterload may be responsible for increased cardiac index with AM. On the other hand, a previous binding study has shown abundant, specific binding sites for AM in ventricular myocardium [37]. AM has been shown to increase cardiac cAMP [33], which is known to mediate the positive inotropic action of beta-adrenergic stimulants. Alternatively, AM has been shown to produce a positive inotropic action through cAMP-independent mechanisms [49]. These findings suggest that the increase in cardiac index may be attributable not only to a fall in cardiac afterload but also to the direct positive inotropic action of AM.

Infusion of AM significantly decreased plasma aldosterone, although there was no significant change in plasma renin activity. In vitro, AM has been shown to inhibit Ang II-induced secretion of aldosterone from dispersed rat adrenal zona glomerulosa cells [51]. Therefore, the inhibition of plasma aldosterone by AM was probably due to a direct effect on adrenal gland, as is the case for atrial natriuretic peptide [46].

It appears that a number of similarities in pharmacologic actions, i.e. vasodilatation, cardiac effect, and cAMP pro-

duction, exist between AM and prostacyclin that is used for reducing pulmonary resistance in PPH. Unlike prostacyclin, however, AM has diuretic and natriuretic activities. AM inhibits inflammation and aldosterone production [7,51]. These biological effects may be the advantages of AM over prostacyclin in respect of therapeutic effectiveness. Exogenously administered AM at a pharmacologic level increased plasma cAMP in association with hemodynamic effects. Thus, additional administration of AM may be effective in patients with pulmonary hypertension.

3. Inhalation of AM

The goal of vasodilator therapy for patients with PPH is to reduce pulmonary vascular resistance without producing systemic hypotension, and improve quality of life and survival. We have shown that intravenous administration of AM markedly decreases pulmonary vascular resistance in patients with PPH [28]. Nevertheless, systemically administered AM decreases systemic arterial pressure, which may be harmful in treating patients with PPH. Recently, inhalation of aerosolized prostacyclin and its analogue, iloprost, has been shown to cause pulmonary vasodilation without systemic hypotension in patients with PPH [11,53]. In addition, inhalant application of vasodilators does not impair gas exchange because the ventilation-matched deposition of drug in the alveoli causes pulmonary vasodilation matched to ventilated areas. In clinical settings, inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. Thus, the purpose of this study was to investigate the effects of AM inhalation on hemodynamics and exercise capacity in patients with PPH.

Interestingly, Champion et al. [5] have shown that intratracheal gene transfer of calcitonin gene-related peptide (CGRP), a member of the same peptide family as AM, to bronchial epithelial cells attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. These results raise the possibility that intratracheal delivery of a vasodilator peptide may be sufficient to alter pulmonary vascular function. In fact, inhalation of AM significantly decreased pulmonary vascular resistance in patients with pulmonary hypertension, whereas it did not alter systemic arterial pressure or systemic vascular resistance [26]. The ratio of pulmonary vascular resistance to systemic vascular resistance was significantly reduced by AM inhalation. These results suggest that inhaled AM improves hemodynamics with pulmonary selectivity. This is consistent with earlier findings that inhaled prostacyclin or its analogue, iloprost, acts transepithelially with pulmonary selectivity and improves pulmonary hypertension.

We examined the long-term effects of inhaled AM in monocrotaline (MCT)-induced pulmonary hypertension rats [30]. AM or saline was inhaled as an aerosol using an ultrasonic nebulizer, for 30 min, four times a day. Repeated

inhalation of AM for three weeks markedly decreased mean pulmonary arterial pressure and pulmonary vascular resistance in MCT rats without systemic hypotension. The potent, long-lasting pulmonary vasodilator effect of inhaled AM may contribute to the strong inhibition of the development of pulmonary hypertension. In addition, considering intermittent delivery of AM to the lungs, the chronic effects of inhaled AM appear to go beyond acute pulmonary vasodilation. Inhalation of AM inhibited an increase in the medial wall thickness of peripheral pulmonary arteries of MCT rats. In vitro studies have shown that AM inhibits the migration and proliferation of vascular smooth muscle cells [12,17]. Given the known potent vasoprotective effects of AM such as vasodilation and inhibition of smooth muscle cell migration and proliferation, it is interesting to speculate that AM trapped in the bronchial epithelium or alveoli leaks to the pulmonary arteries to maintain pulmonary vascular integrity in MCT rats. Importantly, Kaplan–Meier analysis demonstrated that the 6-week survival rate for MCT rats treated with aerosolized AM was significantly high (70%) as compared with 10% in those given saline [30]. Thus, treatment with aerosolized AM may be an alternative approach for severe pulmonary hypertension that is refractory to conventional therapy.

We have demonstrated that inhalation of AM has beneficial hemodynamic effects in animals and humans [26,30]. Recently, pulmonary delivery of a dry-powder insulin has been shown to improve glycemic control without adverse pulmonary effects [48]. Although further studies are necessary to maximize the efficiency and reproducibility of pulmonary AM delivery, combining AM inhalation therapy with other modalities that have a different mode of action may have beneficial effects in patients with PPH.

4. Cell-based AM gene transfer

The pulmonary endothelium plays an important role in the regulation of pulmonary vascular tone through the release of vasoactive substances such as nitric oxide and prostacyclin [6]. Dysfunction of the endothelium may play a role in the pathogenesis of pulmonary hypertension including PPH [4]. Thus, pulmonary endothelial cell may be a therapeutic target for the treatment of pulmonary hypertension. Recently, endothelial progenitor cells have been discovered in adult peripheral blood [2]. EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ [8,18,50]. These findings raise the possibility that transplanted EPCs may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. Thus, we investigated whether cell (EPCs)-based AM gene transfer ameliorates MCT-induced pulmonary hypertension in rats.

We obtained EPCs from cultured human umbilical cord blood mononuclear cells and constructed AM plasmid DNA. We used cationic gelatin to produce ionically linked DNA–gelatin complexes. Interestingly, EPCs phagocytosed plasmid DNA–gelatin complexes, which allowed nonviral, highly efficient gene transfer into EPCs [24]. Recently, intravenously administered hematopoietic cells have been shown to be attracted to sites of cerebral injury [39]. Intravenously injected EPCs accumulate in ischemic myocardium after acute myocardial infarction [18]. These findings suggest that progenitor cells have the capability to sense injured tissues. In fact, intravenously administered gene-modified EPCs were incorporated into pulmonary arterioles and capillaries in MCT rats and differentiated mature endothelial cells [25]. MCT injures endothelial cells of small arteries and capillaries in the lungs, resulting in pulmonary hypertension [44]. Taking these findings together, transplanted EPCs may circulate in the blood and attach to injured pulmonary endothelia in MCT rats. Thus, EPCs may serve not only as a vehicle for gene delivery to injured pulmonary endothelia, but also as a tissue-engineering tool in restoring intact pulmonary endothelium. Transplantation of EPCs without gene modification slightly, but significantly decreased pulmonary vascular resistance in MCT rats [25]. EPCs have been shown to express endothelial nitric oxide synthase and produce nitric oxide [24]. We showed that EPCs produce AM even when its gene is not transduced. These results suggest that vasodilator substances secreted from EPCs contribute to improvement in pulmonary hypertension. We also investigated whether transplantation of gene-modified EPCs causes further improvement in pulmonary hemodynamics and survival in MCT rats [25]. Interestingly, EPCs cultured with AM DNA–gelatin complexes markedly secreted AM protein for more than 2 weeks. These results suggest relatively long-lasting AM secretion from EPCs. The consequence of this synthesis in MCT rats was a marked decrease in mean pulmonary arterial pressure and pulmonary vascular resistance. Histological examination revealed that transplantation of AM-expressing EPCs inhibited an increase in medial wall thickness of pulmonary arteries. Expectedly, transplantation of AM-expressing EPCs caused significantly greater improvement in pulmonary hypertension and vascular remodeling than transplantation of EPCs alone. Given the known potent vasoprotective effects of AM such as vasodilation and inhibition of smooth muscle cell proliferation [12,17], it is interesting to speculate that AM secreted from EPCs may act not only as a circulating factor but also as a autocrine/paracrine factor in the regulation of pulmonary vascular tone and vascular remodeling in MCT rats. Importantly, a single transplantation of AM-expressed EPCs improved survival in MCT rats as compared with administration of EPCs alone or culture medium. These results suggest that ex vivo gene transfer into EPCs greatly enhances therapeutic effects of EPCs transplantation. Further studies are necessary to examine whether repeated administration of EPCs produces an even greater effect than single transplantation.

5. Summary

This article described the therapeutic potential of AM for the treatment of pulmonary hypertension. Baseline plasma AM is significantly higher in patients with pulmonary arterial hypertension. Nevertheless, exogenously administered AM at a pharmacologic level induces hemodynamic improvement. This suggests that an additional administration of AM may be effective in patients with pulmonary hypertension. We have demonstrated the effects of three types of AM delivery systems: intravenous administration of AM peptide, inhalation of AM peptide, and cell-based AM gene transfer. Further studies are necessary to examine which delivery system is the best in clinical settings. AM induces potent pulmonary vasodilation and has vasoprotective effects beyond vasodilation. Thus, AM is a promising endogenous peptide for the treatment of pulmonary arterial hypertension.

Acknowledgements

References

[1] Archer S, Rich S. Primary pulmonary hypertension: a vascular biology and translational research "Work in progress". Circulation 2000;102:2781–91.

[2] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:965–7.

[3] Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, et al. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. N Engl J Med 1996;334:296–302.

[4] Budhiraja R, Tuder RM, Hassoun PM. Endothelial dysfunction in pulmonary hypertension. Circulation 2004;109:159–65, Review.

[5] Champion HC, Bivalacqua TJ, Toyoda K, Heistad DD, Hyman AL, Kadowitz PJ. In vivo gene transfer of prepro-calcitonin gene-related peptide to the lung attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. Circulation 2000;101:923–30.

[6] Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, et al. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. N Engl J Med 1992;327:70–5.

[7] Clementi G, Caruso A, Cutuli VM, Prato A, Mangano NG, Amico-Roxas M. Antiinflammatory activity of adrenomedullin in the acetic acid peritonitis in rats. Life Sci 1999;65:PL203–8.

[8] Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 2001;88:167–74.

[9] Heaton J, Lin B, Chang JK, Steinberg S, Hyman A, Lippton H. Pulmonary vasodilation to adrenomedullin: a novel peptide in humans. Am J Physiol 1995;268:H2211–5.

[10] Higenbottam TW, Wheeldon D, Wells FC, Wallwork J. Long-term treatment of primary pulmonary hypertension with continuous intravenous epoprostenol (prostacyclin). Lancet 1984;1:1046–7.

[11] Hoeper MM, Schwarze M, Ehlerding S, Adler-Schuermeyer A, Spickerkoetter E, Niedermeyer J, et al. Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. N Engl J Med 2000;342:1866–70.

[12] Horio T, Kohno M, Kano H, Ikeda M, Yasunari K, Yokokawa K, et al. Adrenomedullin as a novel antimigration factor of vascular smooth muscle cells. Circ Res 1995;77:660–4.

[13] Ichiki Y, Kitamura K, Kangawa K, Kawamoto M, Matsuo H, Eto T. Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. FEBS Lett 1994;338:6–10.

[14] Ishimitsu T, Nishikimi T, Saito Y, Kitamura K, Eto T, Kangawa K, et al. Plasma levels of adrenomedullin, a newly identified hypotensive peptide, in patients with hypertension and renal failure. J Clin Invest 1994;94:2158–61.

[15] Ishizaka Y, Ishizaka Y, Tanaka M, Kitamura K, Kangawa K, Minamino N, et al. Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. Biochem Biophys Res Commun 1994;200:642–6.

[16] Kakishita M, Nishikimi T, Okano Y, Satoh T, Kyotani S, Nagaya N, et al. Increased plasma levels of adrenomedullin in patients with pulmonary hypertension. Clin Sci (London) 1999;96:33–9.

[17] Kano H, Kohno M, Yasunari K, Yokokawa K, Horio T, Ikeda M, et al. Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. J Hypertens 1996;14:209–13.

[18] Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103:634–7.

[19] Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.

[20] Lippton H, Chang J-K, Hao Q, Summer W, Hyman AL. Adrenomedullin dilates the pulmonary vascular bed in vivo. J Appl Physiol 1994;76:2154–6.

[21] Majid DSA, Kadowitz PJ, Coy DH, Navar LG. Renal responses to intra-arterial administration of adrenomedullin in dogs. Am J Physiol 1996;270:F200–5.

[22] McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 1998;393:333–9.

[23] McLaughlin VV, Genthner DE, Panella MM, Rich S. Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. N Engl J Med 1998;338:273–7.

[24] Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000;105:1527–36.

[25] Nagaya N, Kangawa K, Kanda M, Uematsu M, Horio T, Fukuyama N, et al. Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. Circulation 2003;108:889–95.

[26] Nagaya N, Kyotani S, Uematsu M, Ueno K, Oya H, Nakanishi N, et al. Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. Circulation 27 January 2004;109:351–6.

[27] Nagaya N, Nishikimi T, Horio T, Yoshihara F, Kanazawa A, Matsuo H, et al. Cardiovascular and renal effects of adrenomedullin in rats with heart failure. Am J Physiol 1999;276:R213–8.

[28] Nagaya N, Nishikimi T, Uematsu M, Satoh T, Oya H, Kyotani S, et al. Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. Heart 2000;84:653–8.

[29] Nagaya N, Nishikimi T, Yoshihara F, Horio T, Morimoto A, Kangawa K. Cardiac adrenomedullin gene expression and peptide accumulation after acute myocardial infarction in rats. Am J Physiol 2000;278:R1019–26.

[30] Nagaya N, Okumura H, Uematsu M, Shimizu W, Ono F, Shirai M, et al. Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. Am J Physiol Heart Circ Physiol 2003;285:H2125–31.

[31] Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, et al. Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. Circulation 2000;101:498–503.

[32] Nakamura M, Yoshida H, Makita S, Arakawa N, Niinuma H, Hiramori K. Potent and long-lasting vasodilatory effects of adrenomedullin in humans: comparisons between normal subjects and patients with chronic heart failure. Circulation 1997;95:1214–21.

[33] Nishikimi T, Horio T, Yoshihara F, Nagaya N, Matsuo H, Kangawa K. Effect of adrenomedullin on cAMP and cGMP levels in rat cardiac myocytes and nonmyocytes. Eur J Pharmacol 1998;353:337–44.

[34] Nishikimi T, Saito Y, Kitamura K, Ishimitsu T, Eto T, Kangawa K, et al. Increased plasma levels of adrenomedullin in patients with heart failure. J Am Coll Cardiol 1995;26:1424–31.

[35] Nishimatsu H, Suzuki E, Nagata D, Moriyama N, Satonaka H, Walsh K, et al. Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. Circ Res 2001;89:63–70.

[36] Nossaman BD, Feng CJ, Kaye AD, DeWitt B, Coy DH, Murphy WA, et al. Pulmonary vasodilator responses to adrenomedullin are reduced by NOS inhibitors in rats but not in cats. Am J Physiol 1996;270:L782–9.

[37] Owji AA, Smith DM, Coppock HA, Morgan DG, Bhogal R, Ghatei MA, et al. An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. Endocrinology 1995;136:2127–34.

[38] Pasque MK, Trulock EP, Kaiser LR, Cooper JD. Single lung transplantation for pulmonary hypertension: three month hemodynamic follow-up. Circulation 1991;84:2275–9.

[39] Priller J, Flugel A, Wehner T, Boentert M, Haas CA, Prinz M, et al. Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. Nature Med 2001;7:1356–61.

[40] Rademaker MT, Charles CJ, Lewis LK, Yandle TG, Cooper G.J.S., Coy DH, et al. Beneficial hemodynamic and renal effects of adrenomedullin in an ovine model of heart failure. Circulation 1997;96:1983–90.

[41] Reitz BA, Wallwork JL, Hunt SA, Pennock JL, Billingham ME, Oyer PE, et al. Heart–lung transplantation: successful therapy for patients with pulmonary vascular disease. N Engl J Med 1982;306:557–64.

[42] Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al. Primary pumonary hypertension: a national prospective study. Ann Intern Med 1987;107:216–23.

[43] Rich S. Primary pulmonary hypertension. Prog Cardiovasc Dis 1988;31:205–38.

[44] Rosenberg H, Rabinovitch M. Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. Am J Physiol 1988;255:H1484–91.

[45] Rubin LJ, Mendoza J, Hood M, McGoon M, Barst R, Williams WB, et al. Treatment of primary pulmonary hypertension with continuous intravenous prostacyclin (epoprostenol): results of a randomized trial. Ann Intern Med 1990;112:485–91.

[46] Saito Y, Nakao K, Nishimura K, Sugawara A, Okumura K, Obata K, et al. Clinical application of atrial natriuretic peptide in patients with congestive heart failure: beneficial effects on left ventricular function. Circulation 1987;76:115–24.

[47] Sakata J, Shimokubo T, Kitamura K, Nishizono M, Iehiki Y, Kangawa K, et al. Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. FEBS Lett 1994;352:105–8.

[48] Skyler JS, Cefalu WT, Kourides IA, Landschulz WH, Balagtas CC, Cheng SL, et al. Efficacy of inhaled human insulin in type 1 diabetes mellitus: a randomised proof-of-concept study. Lancet 2001;357:331–5.

[49] Szokodi I, Kinnunen P, Tavi P, Weckstrom M, Toth M, Ruskoaho H. Evidence for cAMP-independent mechanisms mediating the effects of adrenomedullin, a new inotropic peptide. Circulation 1998;97:1062–70.

[50] Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–8.

[51] Yamaguchi T, Baba K, Doi Y, Yano K. Effect of adrenomedullin on aldosterone secretion by dispersed rat adrenal zona glomerulosa cells. Life Sci 1995;56:379–87.

[52] Yoshibayashi M, Kamiya T, Kitamura K, Saito Y, Kangawa K, Nishikimi T, et al. Plasma levels of adrenomedullin in primary and secondary pulmonary hypertension in patients <20 years of age. Am J Cardiol 1997;79:1556–8.

[53] Walmrath D, Schneider T, Pilch J, Grimminger F, Seeger W. Aerosolized prostacyclin reduces pulmonary artery pressure and improves gas exchange in the adult respiratory distress syndrome (ARDS). Lancet 1993;342:961–2.

Adrenomedullin Gene Transfer Induces Therapeutic Angiogenesis in a Rabbit Model of Chronic Hind Limb Ischemia

Benefits of a Novel Nonviral Vector, Gelatin

Noriyuki Tokunaga, MD; Noritoshi Nagaya, MD; Mikiyasu Shirai, MD; Etsuro Tanaka, MD;
Hatsue Ishibashi-Ueda, MD; Mariko Harada-Shiba, MD; Munetake Kanda, MD; Takefumi Ito, MD;
Wataru Shimizu, MD; Yasuhiko Tabata, PhD; Masaaki Uematsu, MD; Kazuhiro Nishigami, MD;
Shunji Sano, MD; Kenji Kangawa, PhD; Hidezo Mori, MD

Background—Earlier studies have shown that adrenomedullin (AM), a potent vasodilator peptide, has a variety of cardiovascular effects. However, whether AM has angiogenic potential remains unknown. This study investigated whether 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 charged biodegradable 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 collateral formation and hind limb perfusion were observed in the naked AM group and AM-gelatin group compared with the control group (calf blood pressure ratio: 0.60±0.02, 0.72±0.03, 0.42±0.06, respectively). Interestingly, hind limb perfusion and capillary density of ischemic muscles were highest in the AM-gelatin group, which revealed the highest content of AM in the muscles among the three groups. As a result, necrosis of lower hind limb and thigh muscles was minimal 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 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. (***Circulation.*** 2004;109:526-531.)

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

A drenomedullin (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.
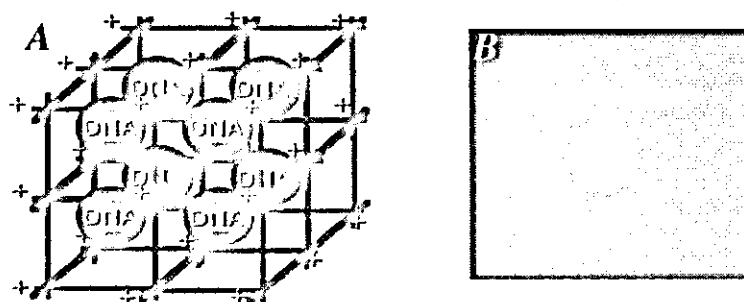
Figure 1. A, Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. B, RITC-labeled AM DNA particles were incorporated into gelatin.

We prepared biodegradable gelatin that could hold negatively charged protein or plasmid DNA in its positively charged lattice structure.[15,16] Biodegradable gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation.[15] Similarly, ionically linked DNA-gelatin complexes can delay gene degradation.[16] 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.[17]

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.[15,16] 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.[16] 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 semimembranous 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, semimembranous, and gastrocnemius muscles were weighed in each hind limb.[18] 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.[17]

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.[17] 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.[17] 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).[19] 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.[20] 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.[21] 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
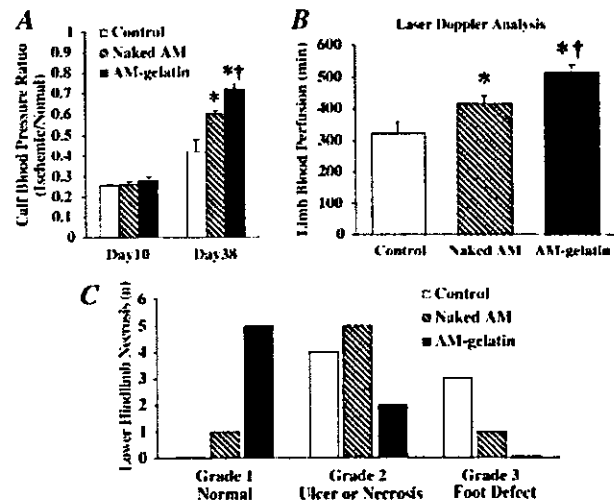

Figure 2. A, Calf blood pressure ratio (ischemic/normal hind limb) before (on day 10) and after (on day 38) gene transfer. B, Measurement of laser Doppler flow on day 38. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. C, Number of cases of each grade of lower hind limb necrosis on day 38. Lower hind limb necrosis was minimal in the AM-gelatin group. Number of necrosis or foot defect is statistically significant among the 3 groups ($P<0.05$ by Kruskal-Wallis test).

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

	Control	Naked AM	AM-Gelatin
No. of rabbits	7	7	7
Body weight, kg	2.46±0.06	2.65±0.10	3.16±0.09
MAP, mm Hg	112±3	114±3	116±2
HR, beats/min	269±12	253±5	262±7
Muscle weight ratio	0.71±0.03	0.84±0.02*	0.95±0.02*†

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.
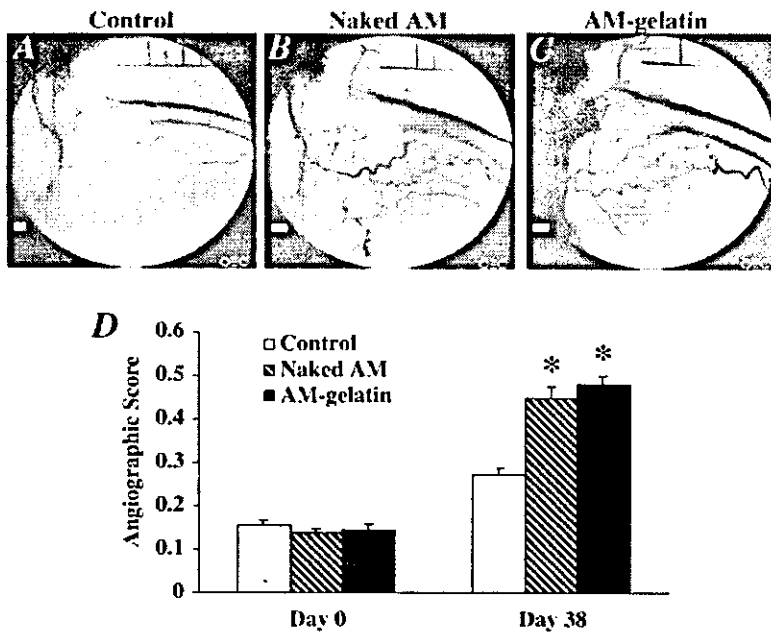*$P<0.01$ vs control group; †$P<0.05$ vs naked AM group.

Figure 3. Representative angiograms of control group (A), naked AM group (B), and AM-gelatin group (C) on day 38. Collateral arteries were well developed in the naked AM and AM-gelatin groups. D, Angiographic score on days 0 and 38 in each group. Angiographic score on day 38 was significantly higher in the naked AM and AM-gelatin groups than in the control group. Data are mean±SEM. *$P<0.001$ versus control 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 phosphory-
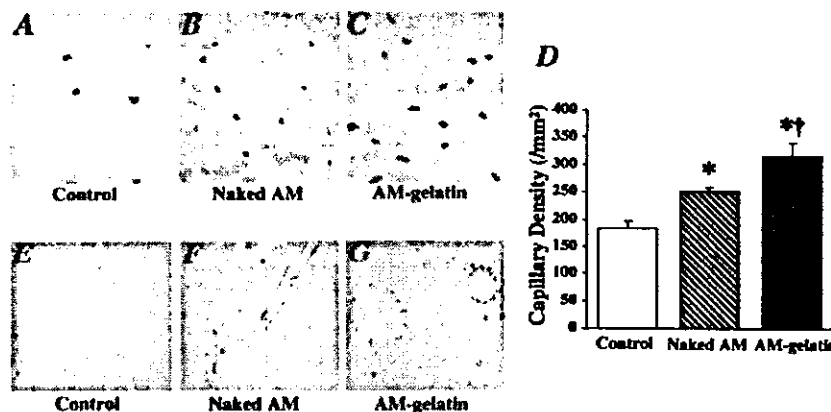

Figure 4. A through C, Representative examples of alkaline phosphatase staining in ischemic hind limb muscles. Magnification ×200. D, Quantitative analysis of capillary density in ischemic hind limb muscles. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. E through G, Immunohistochemical analysis of Ki67 antigen, a marker for cell proliferation. Magnification ×400.
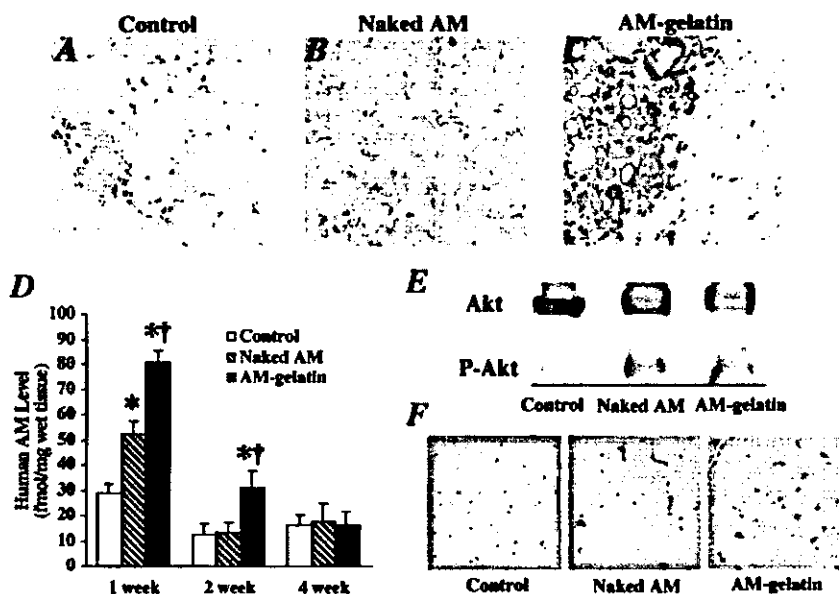
Figure 5. A through C, Immunohisto-chemistry for human AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification ×200. D, Time course of AM production in is-chemic muscles after gene transfer. Data are mean±SEM. *P<0.01 vs control group; †P<0.01 vs naked AM group. E, Western blot analysis for Akt phosphory-lation in muscles. F, Immunohistochemi-cal staining for phosphorylated Akt 7 days after gene transfer. Phosphorylated Akt was distributed at least in endothelial cells. Magnification ×400.

lated Akt was observed at least in endothelial cells of the Naked AM and the AM-gelatin groups (Figure 5F).

Discussion

We demonstrated that (1) AM gene transfer induced hemo-dynamic and angiographic improvements in association with an increase in capillary density in a rabbit model of chronic hind limb ischemia. We also demonstrated that (2) adminis-tration of AM DNA-gelatin complexes markedly augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer.

AM has a variety of effects on the vasculature that include vasodilation,[1,5-7] inhibition of endothelial cell apoptosis,[8,9] and regulation of smooth muscle cell proliferation.[10] How-ever, whether AM has angiogenic potential has remained unknown. In the present study, intramuscular administration of naked AM DNA augmented AM production in skeletal muscles, as indicated by increased tissue content and signif-icant immunostaining of AM. As a result, AM gene transfer increased hind limb perfusion and ameliorated lower hind limb and thigh muscle necrosis in a rabbit model of hind limb ischemia. AM gene transfer may protect the ischemic hind limb partly by improving the blood flow in the ischemic hind limb because AM is originally identified as a potent vasodi-lating peptide.[1] Nevertheless, angiographic collateral devel-opment 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 microves-sels 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 embry-ogenesis.[11-13] These studies support our results that AM may be an angiogenic factor. VEGF is known to induce angiogen-esis and to regulate endothelial cell survival through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[22] Thus, the PI3K/Akt pathway is considered to regulate multiple

critical steps in angiogenesis, including endothelial cell sur-vival, proliferation, migration, and capillary-like structure formation.[14] A recent study has reported that AM promotes proliferation and migration of human umbilical vein endothe-lial cells at least in part through the PI3K/Akt pathway.[21] The present study demonstrated that phosphorylated Akt is in-creased 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 biodegrad-able 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.[15] Thus, biodegradable gelatin has been used as a carrier of protein. However, little infor-mation 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 histolog-ical 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 aug-ments 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-

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.

Acknowledgments

References

1. Kitamura K, Kangawa K, Kawamoto M, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun.* 1993;192:553–560.

2. Sugo S, Minamino N, Kangawa K, et al. Endothelial cells actively synthesize and secrete adrenomedullin. *Biochem Biophys Res Commun.* 1994;201:1160–1166.

3. Sugo S, Minamino N, Shoji H, et al. Production and secretion of adrenomedullin from vascular smooth muscle cells: augmented production by tumor necrosis factor-alpha. *Biochem Biophys Res Commun.* 1994;203:719–726.

4. Kato J, Kitamura K, Kangawa K, et al. Receptors for adrenomedullin in human vascular endothelial cells. *Eur J Pharmacol.* 1995;289:383–385.

5. Shimekake Y, Nagata K, Ohta S, et al. Adrenomedullin stimulates two signal transduction pathways, cAMP accumulation and Ca^{2+} mobilization, in bovine aortic endothelial cells. *J Biol Chem.* 1995;270: 4412–4417.

6. Nagaya N, Satoh T, Nishikimi T, et al. Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation.* 2000;101:498–503.

7. Nishimatsu H, Suzuki E, Nagata D, et al. Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. *Circ Res.* 2001;89:63–70.

8. Kato H, Shichiri M, Marumo F, et al. Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology.* 1997;138:2615–2620.

9. Sata M, Kakoki M, Nagata D, et al. Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis via a cyclic GMP-independent mechanism. *Hypertension.* 2000;36:83–88.

10. Kano H, Kohno M, Yasunari K, et al. Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. *J Hypertens.* 1996; 14:209–213.

11. Shindo T, Kurihara Y, Nishimatsu H, et al. Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation.* 2001;104:1964–1971.

12. Caron KM, Smithies O. Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional adrenomedullin gene. *Proc Natl Acad Sci U S A.* 2001;98:615–619.

13. Imai Y, Shindo T, Maemura K, et al. Evidence for the physiological and pathological roles of adrenomedullin from genetic engineering in mice. *Ann N Y Acad Sci.* 2001;947:26–34.

14. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res.* 2002;90:1243–1250.

15. Tabata Y, Hijikata S, Muniruzzaman M, et al. Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor. *J Biomater Sci Polym Ed.* 1999;10:79–94.

16. Fukunaka Y, Iwanaga K, Morimoto K, et al. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *J Control Release.* 2002;80:333–343.

17. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis: a single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J Clin Invest.* 1994;93: 662–670.

18. Van Belle E, Witzenbichler B, Chen D, et al. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor. *Circulation.* 1998;97:381–390.

19. Ohta H, Tsuji T, Asai S, et al. A simple immunoradiometric assay for measuring the entire molecules of adrenomedullin in human plasma. *Clin Chim Acta.* 1999;287:B131–B143.

20. Nagaya N, Nishikimi T, Yoshihara F, et al. Cardiac adrenomedullin gene expression and peptide accumulation after acute myocardial infarction in rats. *Am J Physiol Regul Integr Comp Physiol.* 2000;278:R1019–R1026.

21. Miyashita K, Itoh H, Sawada N, et al. Adrenomedullin promotes proliferation and migration of cultured endothelial cells. *Hypertens Res.* 2003; 26:S93–S98.

22. Jiang BH, Zheng JZ, Aoki M, et al. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A.* 2000;97: 1749–1753.

23. Tabata Y, Ikada Y. Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres. *J Pharm Pharmacol.* 1987;39:698–704.

24. Nagaya N, Kangawa K, Kanda M, et al. Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. *Circulation.* 2003;108:889–895.