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 $\mu g \cdot kg^{-1} \cdot min^{-1}$) 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.

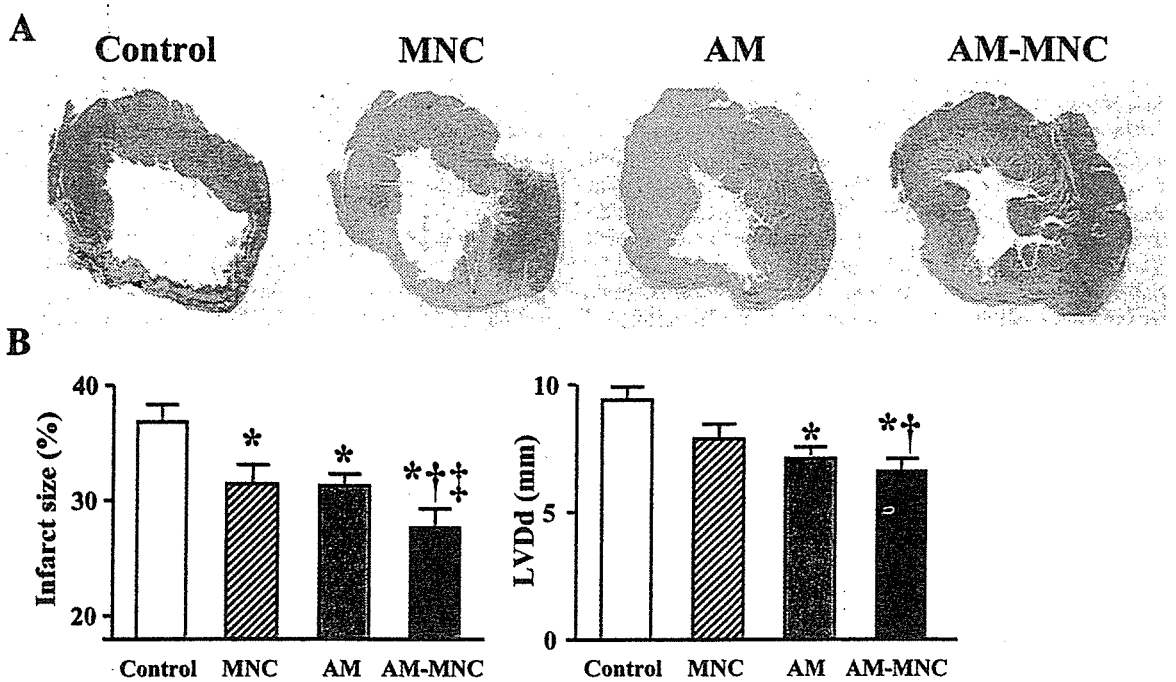

Fig. 1. *A*: representative examples of Masson trichrome-staining of transverse sections of left ventricular (LV) myocardium 4 wk after coronary ligation. *B*: quantitative analysis of infarct size and LV chamber size. Infarcted area and LV end-diastolic diameter (LVDd) of the adrenomedullin-mononuclear cell (AM-MNC) group were significantly smaller than those of the other groups. Values are means ± SE. $*P < 0.05$ vs. control; $\dagger P < 0.05$ vs. MNC; $\ddagger P < 0.05$ 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/d*t*) in the MNC and AM-MNC group were significantly higher than that in the control group. Similarly, LV minimum dP/d*t* 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. 3*A*). Quantitative analysis demonstrated that capillary density was significantly higher in the AM-MNC group than in the MNC and AM groups (Fig. 3*B*). 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; $\dagger P < 0.05$ vs. MNC; $\ddagger P < 0.05$ vs. AM.

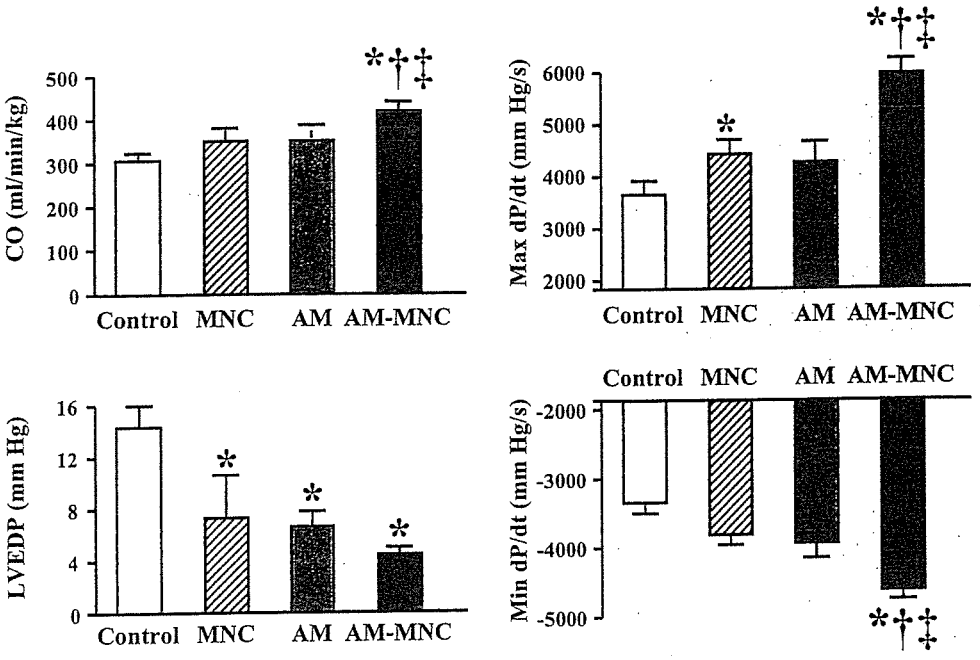

Fig. 2. Effects of AM infusion and MNC transplantation on hemodynamic parameters. CO, cardiac output; LVEDP, LV end-diastolic pressure; Max dP/d*t*, LV maximum change in pressure over time; Min dP/d*t*, LV minimum dP/d*t*. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ 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 ± 1%, $P < 0.05$). However, pretreatment with wortmannin, a PI3-kinase inhibitor, diminished the antiapoptotic effect of AM (17 ± 1%).

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

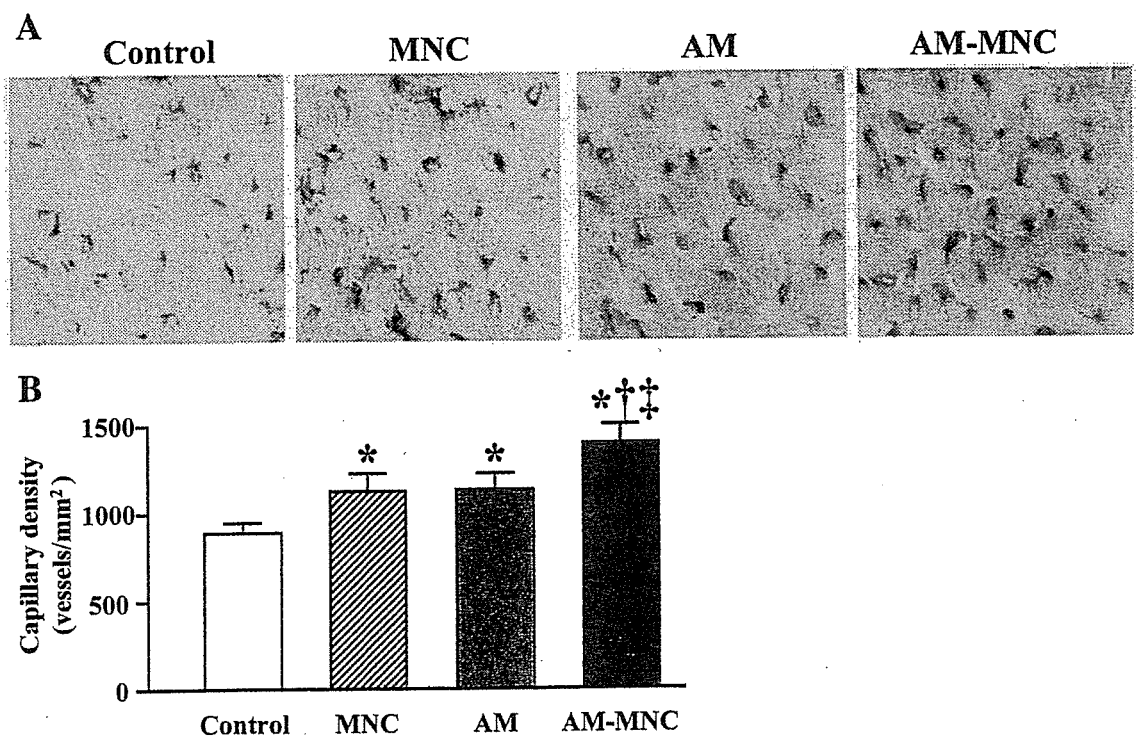

Fig. 3. *A*: representative examples of alkaline phosphatase staining in peri-infarct area. A combination of AM infusion and MNC transplantation markedly induced myocardial neovascularization. Magnification, ×200. *B*: quantitative analysis of capillary density in peri-infarct area. Capillary density 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.

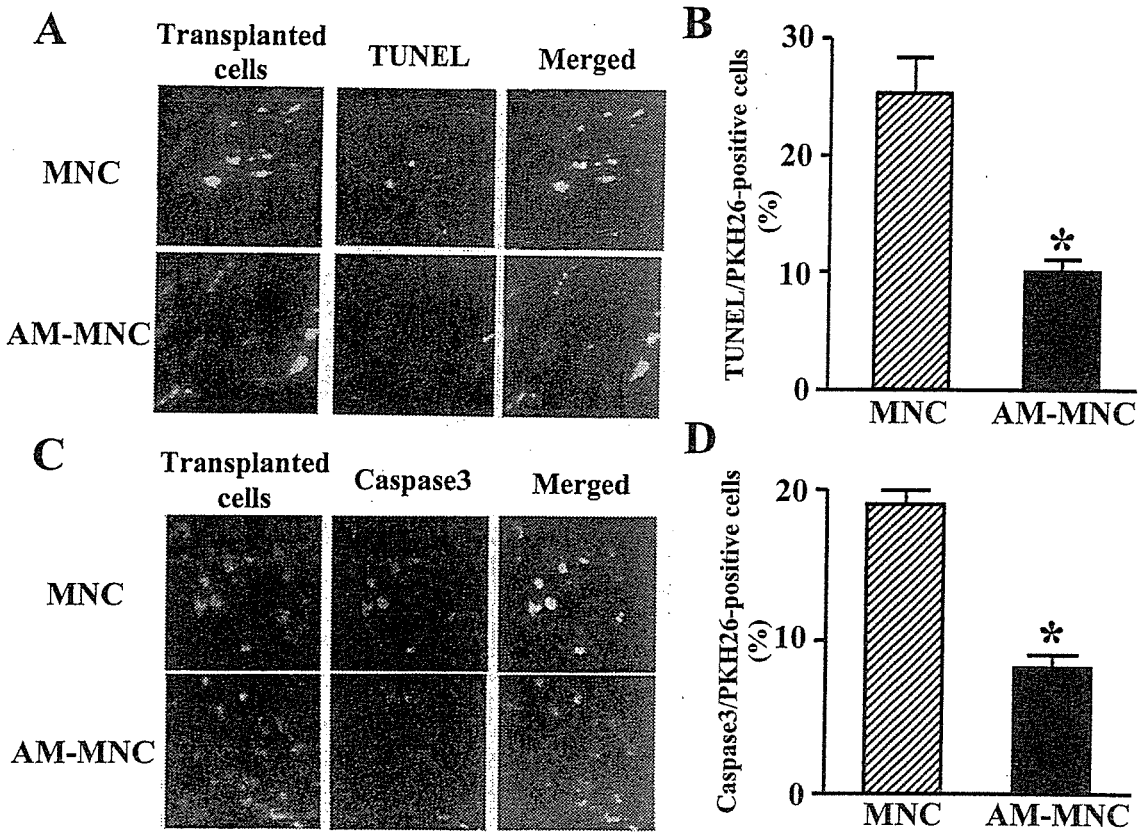

Fig. 4. Detection of transplanted cell apoptosis. *A*: representative photographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates TUNEL-positive cells. TUNEL-positive cells were frequently 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. 5*A*), 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. 5*B*). 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/d*t*. 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

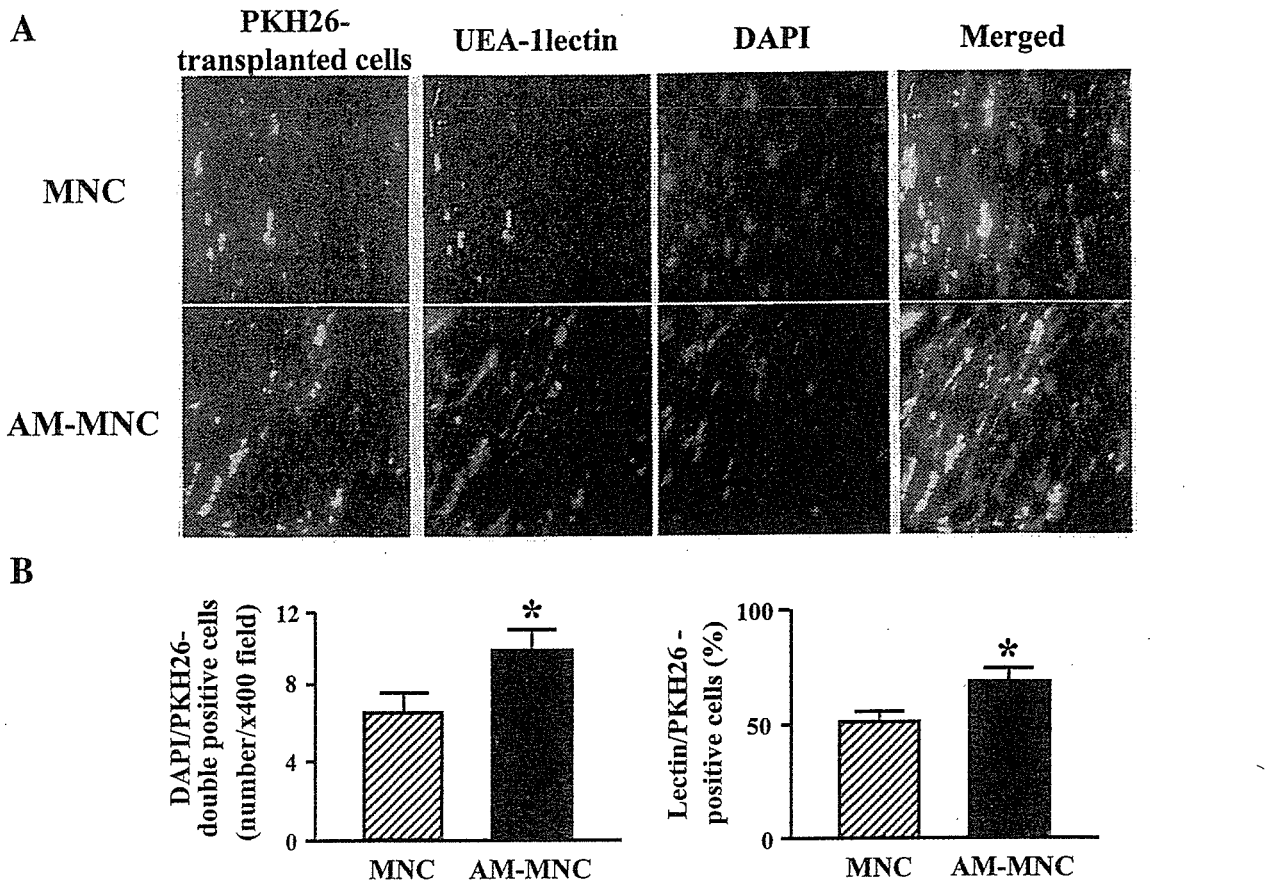

Fig. 5. *A*: representative examples of MNC differentiation into endothelial lineage. Red fluorescence (PKH26) marks transplanted cells; green fluorescence indicates ulex europaeus (UEA)-1 lectin, a marker for vascular endothelial cells. Most of the transplanted cells differentiated into endothelial 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

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Adrenomedullin Enhances Angiogenic Potency of Bone Marrow Transplantation in a Rat Model of Hindlimb Ischemia

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Background—Previous studies have shown that adrenomedullin (AM) inhibits vascular endothelial cell apoptosis and induces angiogenesis. We investigated whether AM enhances bone marrow cell–induced angiogenesis.

Methods and Results—Immediately after hindlimb ischemia was created, rats were randomized to receive AM infusion plus bone marrow–derived mononuclear cell (MNC) transplantation (AM+MNC group), AM infusion alone (AM group), MNC transplantation alone (MNC group), or vehicle infusion (control group). The laser Doppler perfusion index was significantly higher in the AM and MNC groups than in the control group (0.74 ± 0.11 and 0.69 ± 0.07 versus 0.59 ± 0.07, respectively, $P<0.01$), which suggests the angiogenic potency of AM and MNC. Importantly, improvement in blood perfusion was marked in the AM+MNC group (0.84 ± 0.08). Capillary density was highest in the AM+MNC group, followed by the AM and MNC groups. In vitro, AM inhibited MNC apoptosis, promoted MNC adhesiveness to a human umbilical vein endothelial cell monolayer, and increased the number of MNC-derived endothelial progenitor cells. In vivo, AM administration not only enhanced the differentiation of MNC into endothelial cells but also produced mature vessels that included smooth muscle cells.

Conclusions—A combination of AM infusion and MNC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation alone. This effect may be mediated in part by the angiogenic potency of AM itself and the beneficial effects of AM on the survival, adhesion, and differentiation of transplanted MNCs. (*Circulation.* **2005;111:356-362.**)

Key Words: peptides ■ angiogenesis ■ peripheral vascular disease

Peripheral vascular disease is a crucial health issue that affects an estimated 27 million people.[1] Despite recent advances in medical intervention, the symptoms of some patients with critical limb ischemia fail to be controlled. Bone marrow–derived mononuclear cells (MNCs) include a variety of stem and progenitor cells, such as endothelial progenitor cells (EPCs), and contribute to pathological neovascularization.[2] MNC transplantation induces therapeutic angiogenesis in ischemic limb[3,4]; however, some patients fail to respond to this cell therapy. Thus, a novel therapeutic strategy to enhance the angiogenic property of MNCs is desirable.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[5] Previous studies have reported that abnormalities of vascular structure are present in homozygous AM knockout mice.[6,7] A recent study has demonstrated that blood flow recovery in ischemic limb and tumor angiogenesis are substantially impaired in heterozygous AM knockout mice.[8] Furthermore, AM has been shown to inhibit vascular endothelial cell apoptosis and induce angiogenesis through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[9,10] These results suggest that AM is indispensable for modulating angiogenesis and vasculogenesis. When these findings are taken together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of severe peripheral vascular disease. Thus, the purposes of the present study were (1) to investigate whether local infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of hindlimb ischemia and (2) to investigate the effects of AM on the survival, adhesion, and differentiation of transplanted MNCs.

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Methods

Animal Model of Hindlimb Ischemia

Male Lewis rats (weight 250 to 275 g; Japan SLC Inc, Hamamatsu, Japan) were used in the present study. The left common iliac artery of each rat was resected under anesthesia with pentobarbital sodium (50 mg/kg). The distal portion of the saphenous artery and all side branches and veins were dissected free and excised. The right hindlimb was kept intact and used as the nonischemic limb. Transplantation of bone marrow–derived MNCs and infusion of AM were performed in 40 rats immediately after hindlimb ischemia was created. This protocol resulted in the creation of 4 groups: (1) AM infusion plus MNC transplantation (AM+MNC group, n=10), (2) AM infusion plus PBS injection (AM group, n=10), (3) vehicle infusion plus MNC transplantation (MNC group, n=10), and (4) vehicle infusion plus PBS injection (control group, n=10). The Animal Care Committee of the National Cardiovascular Center approved this experimental protocol.

MNC Transplantation and AM Infusion

Bone marrow was harvested from the femur and tibia in other male Lewis rats, and MNCs were isolated by Ficoll density gradient centrifugation (Lymphoprep, Nycomed). MNCs (5×10^6 cells per animal) or PBS was injected into the ischemic thigh muscle with a 26-gauge needle at 5 different points. Human recombinant AM (0.01 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or vehicle was administered for 7 days with a mini-osmotic pump (ALZET, Palo Alto) implanted in the left inguinal region.

Assessment of Blood Perfusion

To measure serial blood flow for 3 weeks, we used a laser Doppler perfusion image (LDPI) analyzer (Moor Instrument). After blood flow was scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion.[11]

Histological Assessment

Three weeks after MNC transplantation and/or AM infusion, 4 pieces of ischemic tissue from the adductor and semimembranous muscles were obtained and snap-frozen in liquid nitrogen. Frozen tissue sections were stained with alkaline phosphatase by an indoxyl tetrazolium method to detect capillary endothelial cells.[3,11] Five fields were randomly selected to count the number of capillaries. The capillary number adjusted per muscle fiber was used to compare the differences in capillary density among the 4 groups.[3]

Monitoring of Transplanted MNCs in Ischemic Hindlimb Muscle

To examine differentiation of transplanted MNCs, 5×10^6 MNCs labeled with red fluorescent dye (PKH26-GL, Sigma Chemical Co) were transplanted into the ischemic thigh muscle in rats with (n=3) and without (n=3) AM infusion. Three weeks after transplantation, frozen tissue sections from ischemic muscle were incubated with anti-von Willebrand factor antibody (vWF, DAKO), anti-CD31 antibody (BD Pharmingen), and anti-α-smooth muscle actin antibody (α-SMA, DAKO), followed by incubation with Alexa Fluor 633 IgG antibody (Molecular Probes) and FITC-conjugated IgG antibody (BD Pharmingen), respectively. Five high-power fields (40×) of each section were randomly selected to count the number of transplanted MNCs, vWF-positive cells, and α-SMA–positive cells.

In Situ Detection of MNC Apoptosis

PKH26-labeled MNCs (5×10^6 cells per animal) were transplanted into the ischemic muscle in rats with (n=2) and without (n=2) AM infusion. Twenty-four hours after transplantation, apoptosis of transplanted MNCs in ischemic tissue was evaluated by terminal dUTP nick-end labeling (TUNEL) assay (ApopTag Fluorescein kit, Serological Corporation), as reported previously.[12]

In Vitro Apoptosis Assay

The antiapoptotic effect of AM on MNCs was evaluated by TUNEL assay. Human MNCs, isolated from peripheral blood, were plated on 12-well plates (1×10^6 cells per well) and cultured in serum-free medium for 24 hours with control buffer, AM, or AM plus wortmannin, a PI3K inhibitor (50 nmol/L). TUNEL for detection of apoptotic nuclei was performed according to the manufacturer's instructions. MNCs were then mounted in medium that contained 4′,6-diamidino-2-phenylindole (DAPI). Randomly selected microscopic fields (n=10) were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

Adhesion Assay

We evaluated whether AM enhances MNC adhesiveness according to a previously reported method.[13] In brief, human umbilical vein endothelial cells (HUVECs) were cultured to confluence on 6-well plates with or without pretreatment with tumor necrosis factor-α (1 ng/mL). In the absence or presence of AM (10^{-7} mol/L), 1×10^6 MNCs labeled with PKH26 were incubated on an HUVEC monolayer for 24 hours. Nonadherent MNCs were removed, and the number of PKH26-positive cells in each well was counted.

Cell ELISA

Expression of adhesion molecules in HUVECs was measured by cell ELISA, as reported previously.[14] In brief, confluent HUVECs on 96-well plates were treated with AM (10^{-7} mol/L) or control buffer for 4 hours. HUVECs were then incubated with monoclonal mouse antibodies against intercellular adhesion molecule-1 (ICAM-1, R&D Systems) and vascular adhesion molecule-1 (VCAM-1, R&D Systems). A protein detector ELISA kit (KPL) was used to detect bound monoclonal antibodies.

EPC Culture Assay

Culture of EPCs was performed as described previously.[11,15,16] In brief, 2×10^6 MNCs were plated in Medium-199 supplemented with 20% FCS, heparin, and antibiotics on fibronectin-coated 6-well plates. AM (10^{-7} mol/L), human recombinant vascular endothelial growth factor (VEGF; 20 ng/mL), or control buffer was added to each plate. After 7 days of culture, nonadherent cells were removed, and adherent cells were incubated with acetylated LDL labeled with DiI (DiI-acLDL, Biomedical Technologies) and FITC-labeled lectin from ulex europaeus (Sigma). Double-positive cells for DiI-acLDL and FITC-labeled lectin were identified as EPCs.[16] Randomly selected microscopic fields (n=10) were evaluated to count the number of EPCs.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting was performed to identify characteristics of adherent cells after 7 days of culture.[16] Cells were incubated for 30 minutes at 4°C with anti-human CD31 antibodies (clone L133.1, Becton Dickinson), anti-human KDR antibodies (clone KDR-1, Sigma), and anti-human VE-cadherin antibodies (clone BV6, Chemicon). Isotype-identical antibodies served as controls. Fluorescence-activated cell sorting analyses were performed with a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences).

Real-Time Polymerase Chain Reaction

Expression of calcitonin receptor-like receptor (CRLR), a receptor for AM, was examined by real-time polymerase chain reaction (PCR). Total RNA was extracted from MNCs, EPCs, and HUVECs with an RNA extraction kit (RNeasy Mini Kit, Qiagen) and converted to cDNA by reverse transcription. Real-time PCR was performed with SYBR green dye (QuantiTect SYBR Green PCR kit, Qiagen) and a Prism 7700 sequence detection system (Applied Biosystems). The PCR primers for CRLR were as follows: sense primer 5′-CATTCAACAAGCAGAAGGCG-3′ and antisense primer 5′-AGCCATCCATCCCAGGTTC-3′. For GAPDH, the primers were as follows: sense primer 5′-CAATGCCTCCTGCACCACCAA-3′ and antisense primer 5′-GAGGCAGGGATGATGTTCTGGA-3′. Levels of CRLR mRNA were normalized to that of

GAPDH mRNA. PCR-amplified products were also electrophoresed on 2% agarose gels to confirm that single bands were amplified.

In Vitro Matrigel Assay

HUVECs (1×10^5 cells) were seeded onto 24-well plates coated with Matrigel (Becton Dickinson) in the presence of the combination of control buffer, AM (10^{-7} mol/L), VEGF (10 ng/mL), or neutralizing antibodies against KDR (2 μg/mL, R&D Systems). After incubation for 18 hours, tube formation area was measured as described previously.[17] The control was defined as 100% tube formation, and the percent increase was calculated for each sample.

Measurements of Cytokines

A total of 1×10^6 MNCs or HUVECs were plated in serum-free medium with or without AM (10^{-7} mol/L) on 12-well plates. After 24-hour incubation, the conditioned medium was collected, and VEGF, basic fibroblast growth factor, and hepatocyte growth factor were measured with enzyme immunoassay kits (R&D Systems).

Migration Assay

Migration assay of smooth muscle cells (SMCs) was performed with Transwell (Coster) 24-well plates composed of a collagen-coated membrane with 8-μm pores. Human aortic SMCs, preincubated with serum-free medium for 24 hours to maintain quiescence, were seeded on the upper chamber at a concentration of 1×10^6 cells/mL. Serum-free medium containing control buffer, AM (10^{-7} mol/L), or AM plus wortmannin (50 nmol/L) was placed in the lower chamber. After incubation for 12 hours, the number of migrated cells was counted in the randomly selected fields (n=5).

Statistical Analysis

All values are expressed as mean±SEM. Student's unpaired *t* test was used to compare differences between 2 groups. Comparisons of parameters among 3 or 4 groups were made by 1-way ANOVA, followed by Scheffé multiple comparison test. Comparisons of the time course of the LDPI index were made by 2-way ANOVA for repeated measures, followed by Scheffé multiple comparison tests. A probability value <0.05 was considered statistically significant.

Results

Blood Perfusion and Capillary Density

Blood perfusion of the ischemic hindlimb increased modestly but gradually in the AM and MNC groups after treatment (Figure 1A). Interestingly, blood perfusion in the AM+MNC group markedly improved within 2 weeks after treatment and showed further improvement thereafter. The LDPI index was significantly higher in the AM, MNC, and AM+MNC groups than in the control group 3 weeks after surgery (Figure 1B). Importantly, the LDPI index was highest in the AM+MNC group among the 4 groups.

Alkaline phosphatase staining of ischemic muscle showed significant augmentation of neovascularization in the AM, MNC, and AM+MNC groups (Figure 2A). The capillary/muscle fiber ratio of ischemic muscle was highest in the AM+MNC group, followed by the MNC group, AM group, and control group (Figure 2B).

Differentiation of Transplanted MNCs

Three weeks after MNC transplantation, PKH26-labeled MNCs were frequently observed in the AM+MNC group, and these transplanted cells were positive for vWF (Figure 3A). Most of these cells were also stained by CD31 (data not shown). The number of PKH26/vWF double-positive cells was significantly higher in the AM+MNC group than in the MNC group (Figure 3B). Although PKH26/α-SMA double-positive cells were not detected in ischemic muscle of each group, newly formed vascular structures in the AM+MNC group included α-SMA–positive cells (Figure 3C). The number of α-SMA–positive cells in the MNC-derived vascular structures was significantly higher in the AM+MNC group than in the MNC group (Figure 3D).

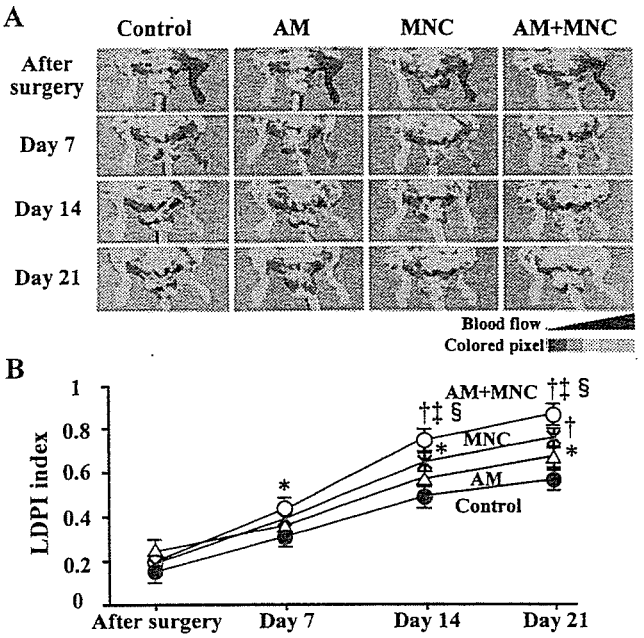

Figure 1. A, Representative examples of serial laser Doppler perfusion images. Blood perfusion of ischemic hindlimb increased notably in AM+MNC group (red to yellow). B, Quantitative analysis of hindlimb blood perfusion with LDPI index, ratio of ischemic to nonischemic hindlimb blood perfusion. Data are mean±SEM. *$P<0.05$ and †$P<0.01$ vs control; ‡$P<0.01$ vs AM; §$P<0.05$ vs MNC.

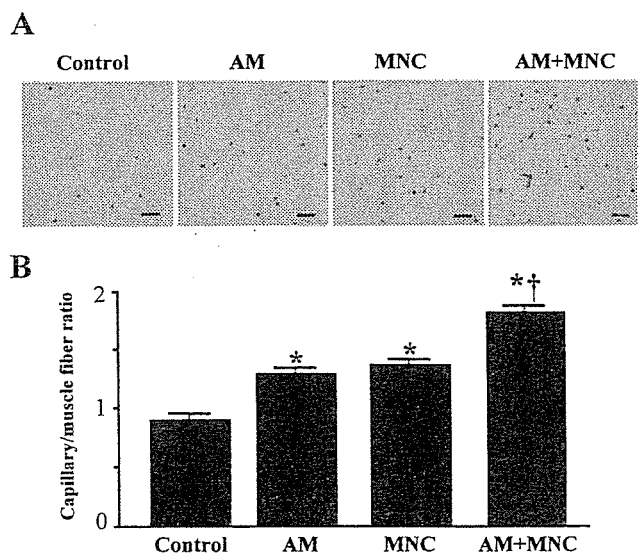

Figure 2. A, Representative photographs of alkaline phosphatase staining in ischemic hindlimb muscles. Capillary density in AM+MNC group was markedly higher than that in other groups. B, Quantitative analysis of capillary density in ischemic hindlimb muscles. Data are mean±SEM. *$P<0.01$ vs control; †$P<0.01$ vs AM and MNC. Scale bars: 50 μm.

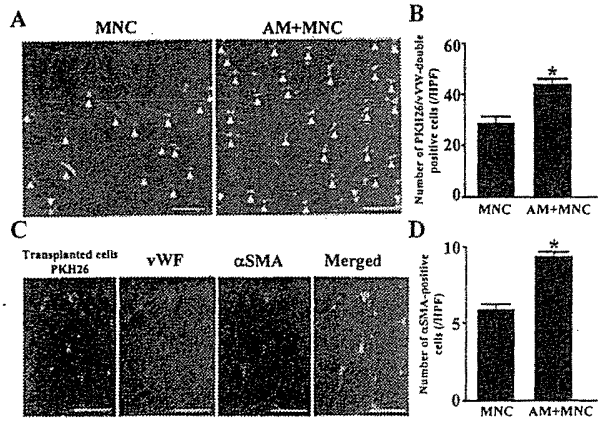

Figure 3. In vivo differentiation of transplanted MNCs. A, Representative photographs of MNC-derived vascular structures in MNC and AM+MNC groups. Red fluorescence (PKH26)–labeled MNCs were transplanted into ischemic thigh muscle. PKH26 (red)/vWF (blue) double-positive cells (pink, arrows) were frequently observed in AM+MNC group. B, Number of PKH26/vWF double-positive cells (MNC-derived endothelial cells) was significantly higher in AM+MNC group than in MNC group. C, Representative photographs of newly formed mature vessels in AM+MNC group. MNC-derived vascular structures often included α-SMA–positive cells (green). D, Number of α-SMA–positive cells in MNC-derived vessels was significantly higher in AM+MNC group than in MNC group. Data are mean±SEM. *P<0.01 vs MNC. Bars: 50 μm. HPF indicates high-power field.

Antiapoptotic Effect of AM on MNCs

In vitro, serum starvation induced MNC apoptosis, as indicated by detection of TUNEL-positive cells (Figure 4A). When incubated in the presence of AM, the percentage of TUNEL-positive cells markedly decreased in a dose-dependent manner (Figure 4B). However, pretreatment with wortmannin, a PI3K inhibitor, diminished the antiapoptotic effect of AM. Similarly, in vivo, local administration of AM decreased TUNEL-positive MNC 24 hours after transplantation (data not shown).

Effect of AM on MNC Adhesiveness

The number of adherent MNCs on an HUVEC monolayer increased significantly in the presence of AM (10^{-7} mol/L) compared with control (Figures 5A and 5B). With pretreatment using tumor necrosis factor-α, AM also enhanced the adhesiveness of MNCs to HUVECs. AM significantly enhanced expression of ICAM-1 and VCAM-1 in HUVECs (Figure 5C).

Effect of AM on EPC Expansion

After 7-day culture of human MNCs, spindle-shaped or cobblestone-like adherent cells were observed (Figure 6A). Most of the adherent cells were double stained with DiI-acLDL and FITC-labeled lectin. These adherent cells expressed endothelial cell–specific markers: KDR, VE cadherin, and CD31 (Figure 6B). Thus, we identified the major population of the adherent cells as EPCs. Culture of MNCs with AM significantly increased the number of EPCs (Figure 6C). The effect of AM was equivalent to that of VEGF. Real-time PCR revealed that MNCs, EPCs, and HUVECs expressed mRNA of CRLR (Figure 6D). Expression of

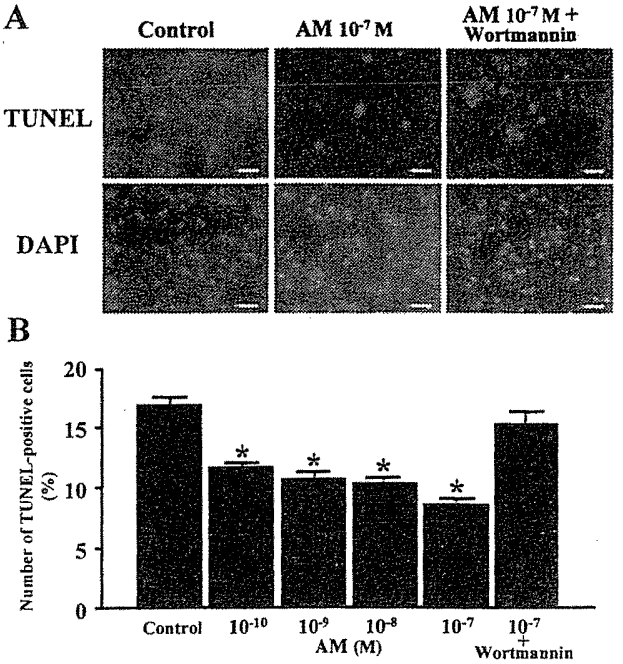

Figure 4. Apoptosis assay. A, Apoptosis of MNC was detected by TUNEL assay (green). Nuclei of MNC were stained with DAPI (blue). AM inhibited MNC apoptosis in serum-free medium. B, Quantitative analysis. AM decreased percentage of TUNEL-positive cells in dose-dependent manner. Pretreatment with wortmannin, a PI3K inhibitor, diminished antiapoptotic effect of AM. Data are mean±SEM. *P<0.01 vs control. Bars: 50 μm.

CRLR mRNA was highest in HUVECs, followed by EPCs and MNCs.

Effects of AM on Tube Formation and SMC Migration

Like VEGF, AM induced tube formation in HUVECs in vitro (Figure 7A). Blocking antibodies against KDR significantly inhibited VEGF-induced tube formation, whereas they did not suppress AM-induced tube formation (Figure 7B). AM did not significantly alter VEGF, basic fibroblast growth factor, or hepatocyte growth factor levels in conditioned medium of cultured MNCs or HUVECs (data not shown). AM significantly increased the number of migrated SMCs compared with control (Figures 7C and 7D). Pretreatment with wortmannin diminished the effect of AM on SMC migration.

Discussion

In the present study, we demonstrated in vivo that AM infusion or MNC transplantation alone induced angiogenesis in a rat model of hindlimb ischemia, the combination of AM infusion and MNC transplantation enhanced MNC-induced angiogenesis, and AM increased the number of MNC-derived vWF-positive cells and generated α-SMA–positive vascular structures. We also demonstrated in vitro that AM inhibited serum starvation–induced MNC apoptosis, promoted MNC adhesiveness to an HUVEC monolayer, increased the number of MNC-derived EPCs, and stimulated SMC migration.

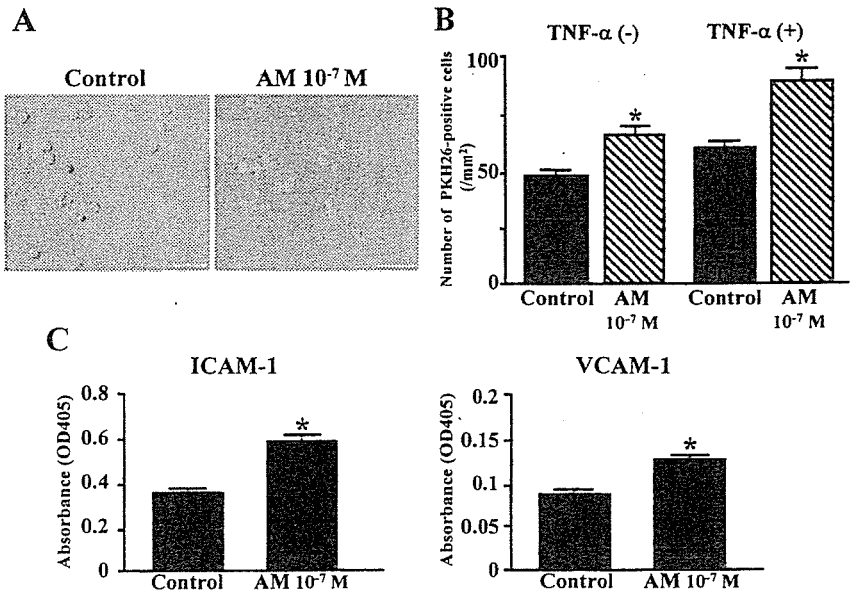

Figure 5. A and B, Adhesion assay. Representative photographs of red fluorescence–labeled MNC adhesion to HUVEC monolayer with and without AM (A). Quantitative analysis of MNC adhesion (B). Bars: 50 μm. C, Surface expression of ICAM-1 and VCAM-1 in HUVECs with or without AM. Data are mean±SEM. TNF indicates tumor necrosis factor. *$P<0.01$ vs control.

MNC transplantation causes therapeutic angiogenesis by supplying EPCs and multiple angiogenic cytokines such as VEGF.[3,4] The present study showed that local infusion of AM significantly increased blood perfusion and capillary density in ischemic hindlimb muscle. Furthermore, a combination of AM infusion and MNC transplantation significantly increased blood perfusion and capillary density of the ischemic hindlimb compared with MNC transplantation alone. AM has been shown to induce angiogenesis in vitro and in vivo through the PI3K/Akt pathway.[10,18] In the present study, AM-induced tube formation was not blocked by neutralizing antibodies against KDR. In addition, AM did not enhance VEGF secretion from MNCs and HUVECs. Thus, beneficial effects of combination therapy

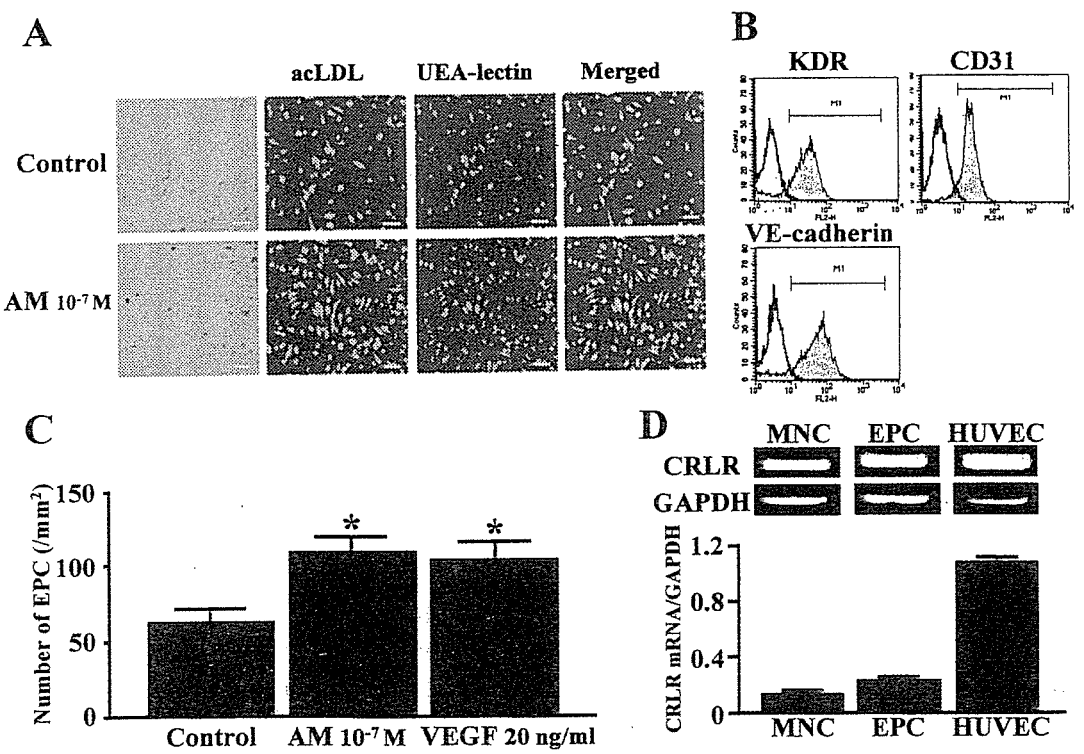

Figure 6. A through C, EPC culture assay. Cultured adherent cells took up DiI-acLDL (red) and FITC-labeled lectin (green) in same fields (A). Fluorescence-activated cell sorting analyses revealed that most adherent cells expressed KDR, VE cadherin, and CD31 (B). Culture of MNCs with AM significantly increased number of EPCs. Effect of AM was equivalent to that of VEGF (C). Data are mean±SEM. *$P<0.01$ vs control. Bars: 50 μm. D, Quantitative analysis of AM receptor (CRLR) mRNA expression in MNCs, EPCs, and HUVECs. UEA indicates ulex europaeus.

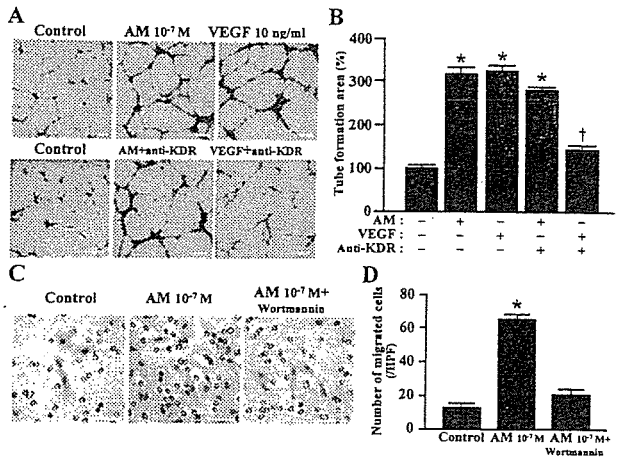

Figure 7. A and B, Matrigel assay. Representative photographs of tube formation (A). Quantitative analysis of tube formation area (B). Data are mean±SEM. *P<0.01 vs control; †P<0.01 vs VEGF. Bars: 20 μm. C and D, Migration assay. Representative photographs of migrated SMCs (C). Quantitative analyses of SMC migration (D). Data are mean±SEM. *P<0.01 vs control. Bars: 50 μm.

with AM and MNCs may be attributable in part to the angiogenic properties of AM itself.

An earlier study has shown that transplanted MNCs disappear from ischemic muscle 7 days after transplantation.[19] We demonstrated that apoptosis of MNCs occurred in ischemic muscle 24 hours after MNC transplantation. These results raise the possibility that the angiogenic potency of MNC transplantation is attenuated by MNC apoptosis. In the present study, AM inhibited apoptosis of MNCs in vitro and in vivo, and the antiapoptotic effect of AM was suppressed by wortmannin, a PI3K inhibitor. These findings suggest that AM prolongs MNC survival through the PI3K/Akt pathway and thereby enhances neovascularization in ischemic tissue.

In the present study, AM promoted adhesiveness of MNCs to an HUVEC monolayer. AM significantly enhanced expression of ICAM-1 and VCAM-1 in HUVECs, both of which facilitate adhesion of MNCs to endothelial cells.[20] These findings suggest that AM increases MNC adhesiveness to endothelial cells via activation of adhesion molecules. A recent study has shown that MNC adhesiveness to endothelial cells is indispensable for MNC differentiation into endothelial lineage.[21] Thus, it is possible that AM infusion enhances the angiogenic potency of MNCs at least in part through promotion of adhesion of MNC to host vascular endothelial cells.

VEGF has been shown to increase the number of EPCs in vitro and in vivo, resulting in angiogenesis and vasculogenesis.[13,22] The present study showed that MNCs and EPCs expressed CRLR, a receptor of AM. In vitro, AM increased the number of MNC-derived EPCs that expressed VE cadherin, KDR, and CD31. The effect of AM on EPC expansion was equivalent to that of VEGF. In vivo, AM infusion increased the number of MNC-derived vWF-positive cells, although incorporation of these cells in the capillaries may be due in part to incorporation of hematopoietic cells. These findings suggest that AM may accelerate MNC differentiation into endothelial lineage.

SMC is essential for the generation of functional and mature blood vessels.[23] We demonstrated in vivo that local infusion of AM increased the number of α-SMA–positive cells (SMCs) in MNC-derived vascular structures. In vitro, AM enhanced SMC migration, which was inhibited by wortmannin, a PI3K inhibitor. Recent studies using homozygous AM knockout mice have suggested that AM is indispensable for vascular morphogenesis.[6,7] When these findings are taken together, it is possible that AM contributes to vessel maturation through enhancement of SMC migration via the PI3K/Akt-dependent pathway.

Currently, a new therapeutic approach to augment the efficacy of MNC transplantation is awaited for the treatment of severe peripheral vascular disease. The present study demonstrated that local infusion of AM enhanced the angiogenic potency of MNC transplantation. In the present study, AM inhibited MNC apoptosis and increased the total number of engrafted cells in ischemic tissue, although this study did not show the effect of AM on specific cell populations of MNCs. In addition, AM promoted cell proliferation, migration, and differentiation. We have already demonstrated the safety of AM infusion in patients with congestive heart failure.[24] Thus, combination therapy with AM infusion and MNC transplantation may be a novel and promising therapeutic strategy for the treatment of severe peripheral vascular disease.

Conclusions

A combination of AM infusion and MNC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation alone. This effect may be mediated in part by the angiogenic potency of AM itself and the beneficial effects of AM on the survival, adhesion, and differentiation of transplanted MNCs.

Acknowledgments

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Adrenomedullin Enhances Therapeutic Potency of Mesenchymal Stem Cells After Experimental Stroke in Rats

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Background and Purpose—Adrenomedullin (AM) induces angiogenesis and inhibits cell apoptosis through the phosphatidylinositol 3-kinase/Akt pathway. Transplantation of mesenchymal stem cells (MSCs) has been shown to improve neurological deficits after stroke in rats. We investigated whether AM enhances the therapeutic potency of MSC transplantation.

Methods—Male Lewis rats (n=100) were subjected to 2-hour middle cerebral artery occlusion. Immediately after reperfusion, rats were assigned randomly to receive intravenous transplantation of MSCs plus subcutaneous infusion of AM for 7 days (MSC+AM group), AM infusion alone (AM group), MSC transplantation alone (MSC group), or vehicle infusion (control group). Neurological and immunohistological assessments were performed to examine the effects of these treatments.

Results—Some engrafted MSCs were positive for neuronal and endothelial cell markers, although the number of differentiated MSCs did not differ significantly between the MSC and MSC+AM groups. The neurological score significantly improved in the MSC, AM, and MSC+AM groups compared with the control group. Importantly, improvement in the MSC+AM group was significantly greater than that in the MSC and AM groups. There was marked induction of angiogenesis in the ischemic penumbra in the MSC+AM group, followed by the AM, MSC, and control groups. AM infusion significantly inhibited apoptosis of transplanted MSCs. As a result, the number of engrafted MSCs in the MSC+AM group was significantly higher than that in the MSC group.

Conclusions—AM enhanced the therapeutic potency of MSCs, including neurological improvement, possibly through inhibition of MSC apoptosis and induction of angiogenesis. (***Stroke.* 2005;36:853-858.**)

Key Words: angiogenesis ■ apoptosis ■ stroke

Despite the advances in medical and surgical treatment, stroke is still a major cause of morbidity and mortality. Mesenchymal stem cells (MSCs) are multipotent, and some transplanted MSCs can differentiate into neuronal cells and endothelial cells in the recipient brain.[1] A recent study has shown that MSCs have ability to pass blood–brain barrier, particularly in injury sites.[1–3] In addition, transplantation of MSCs into the brain of experimental stroke animals has been shown to improve neurological functional recovery.[1,3] The effect of MSC transplantation is dependent on the number of transplanted MSCs.[1] However, the viability of MSCs after transplantation is relatively poor.[4] Thus, a new approach to augment the effect of MSC transplantation is desirable for the application of MSC therapy to the regenerative treatment of stroke.

Adrenomedullin (AM) is a potent vasodilatory peptide that was originally isolated from human pheochromocytoma.[5] Recent study has shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a hindlimb ischemic model via activation of Akt.[6] In addition, AM has been shown to exert antiapoptotic effects on a variety of cells.[7] We also demonstrated antiapoptotic effects of AM in myocardial ischemia/reperfusion injury through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[8] These results suggest that AM may play an important role in induction of angiogenesis and inhibition of apoptosis. Taking these findings together, AM infusion may have additive or synergetic effects on MSC transplantation, which may result in improvement of neurological functional recovery. Thus, the purpose of this study was to investigate whether combined therapy of AM infusion and MSC transplantation significantly improves neurological functional recovery compared with MSC transplantation alone.

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Materials and Methods

Stroke Model

Male Lewis rats (Japan SLC, Hamamatsu, Japan) weighing 230 to 260 g were used in all experiments. Middle cerebral artery occlusion (MCAO) was performed by an intraluminal thread as described previously.[2] The animal care committee of the National Cardiovascular Center approved this experimental protocol.

MSC Preparation

MSC expansion was performed according to a previously described method.[9] In brief, we euthanized male Lewis rats and harvested bone marrow. Bone marrow cells were introduced into 100-mm dishes and cultured in α-minimum essential medium (MEN) supplemented with 10% FBS. After nonadherent hematopoietic cells were removed with medium replacement, spindle-shaped adherent cells developed visible symmetric colonies by day 5 to 7. They were expanded to >50 million cells, ≈4 to 5 passages. These adherent cells were collected with 0.05% trypsin and 2% EDTA (GIBCO) for 3 minutes at 37°C. These cells were analyzed by fluorescence-activated cell sorting as described previously.[10] Most of cultured adherent cells were positive for CD29 (98±1%) and CD90 (99±1%) and negative for CD34 (2±1%) and CD45 (1±1%). We confirmed that major population of the adherent cells were MSCs. MSCs secreted a large amount of an antiapoptotic and angiogenic factor, including vascular endothelial growth factor (VEGF; 960±14 pg/10^6 cells), 24 hours after culture.

MSC Transplantation and AM Infusion

Immediately after 2-hour MCAO, rats were assigned randomly to the following 4 groups. (1) PBS injection plus vehicle infusion (control group n=22); (2) MSC injection plus vehicle infusion (MSC group n=28); (3) PBS injection and AM infusion (AM group n=22); and (4) MSC injection plus AM infusion (MSC+AM group n=28). MSCs (1×10^6 cells) suspended in PBS were injected via a tail vein. Four rats underwent a sham operation without an intraluminal thread. AM (0.05 μg/kg per minute) or vehicle was infused for 7 days using a mini-osmotic pump (Alzet) implanted in the posterior cervical subcutaneous region. The dose of AM used in this study has antiapoptotic effects without significant hypotension.[8]

Detection of MSC Differentiation in Ischemic Hemisphere

Red fluorescent-labeled MSCs were transplanted to examine MSC differentiation as described previously.[11] In brief, suspended MSCs were labeled with fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit; Sigma). Three minutes after labeling, FBS was added for 1 minute to stop reaction and cells were washed by PBS. A recent study has shown that the sensitivity and specificity for cell labeling with PKH26 are ≈100%, and transplanted cells are detectable at least up to 4 months after transplantation in the host brain.[11] Rats were euthanized with an overdose of pentobarbital on day 14 after MCAO. For preparation of frozen sections, rats were perfused transcardially with normal saline and the brain was removed immediately. Blocks corresponding to coronal coordinates for bregma −1 to 1 mm were obtained and frozen rapidly in liquid nitrogen. A series of 6-μm-thick sections was obtained. Numbers of PKH26-positive cells were counted in a blind fashion and expressed as the average in 5 sections. To detect the differentiation of MSCs, immunohistochemical staining was performed. Sections were incubated with anti-von Willebrand factor (vWF) polyclonal antibody (1:200; DAKO, Glostrup, Denmark), rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; DAKO), and mouse anti-neuronal nuclei marker (NeuN; 1:200; Chemicon, Hampshire, UK), followed by incubation with fluorescein isothiocynate (FITC)–conjugated rabbit immunoglobulin antibody (DAKO) and FITC-conjugated mouse immunoglobulin antibody (BD Pharmingen, San Diego, Calif), respectively.

Neurological Assessment

Neurological assessment was performed on days 1, 7, and 14 using a modified neurological severity score, as described previously.[1] In brief, this score is derived by evaluating animals for hemiparesis (response to raising the rat by the tail or placing the rat on a flat surface), sensory deficits (placing, proprioception), beam balance tests (response to placement and posture on a narrow beam and time before dropping), absent reflexes (pinna, corneal, startle), and abnormal movement (seizure, myoclonus, myodystony). One point is awarded for the inability to perform a task or for the lack of a tested reflex.

Measurement of Infarct Size

Rats were euthanized on day 1 (each group n=8) and on day 14 (each group n=8). For preparation of paraffin-embedded sections, rats were perfused transcardially with 4% paraformaldehyde. Brains were cut into 7 equally spaced (2 mm) coronal blocks, and each section was stained with hematoxylin and eosin. Infarct size was determined by the "indirect method," as described previously,[1] and expressed as a percentage of the intact contralateral hemispheric size.

Assessment of Angiogenesis

Angiogenesis was analyzed on day 14 (each group n=8). Paraffin sections corresponding to coronal coordinates for bregma −1 to 1 mm were selected. Sections were incubated with anti-vWF antibody and then incubated with biotinylated anti-rabbit immunoglobulin and with streptoavidin-horseradish peroxidase (HRP) complex (DAKO). The HRP reaction was detected in diaminobenzidine (DAB). To quantify angiogenesis, 8 fields of view from the ischemic penumbra and contralateral noninfarct tissue were randomly selected as described previously,[2] and images (×100 magnification) were acquired using a microscope (ZWISS AXIOVERT 135) and a digital camera (ZWISS AXIO cam). The vWF-immunoreactive area in each image was determined by image analysis using software (Win Roof 5.0; Microsoft) as described previously.[12] The values corresponding to total brown areas were averaged and expressed as the mean percentage of stained vessel area per 100 μm^2. To detect newly formed vessels, tissue sections were stained for Ki67, a marker for cell proliferation, with the use of monoclonal anti-Ki67 antibody (DAKO). The numbers of Ki67-positive microvessels were counted and expressed the average in 8 fields.

Detection of Apoptosis in Ischemic Penumbra

The antiapoptotic effects of AM on the ischemic penumbra were examined 24 hours after MCAO (each group n=8). Paraffin-embedded sections were prepared for TUNEL assay. TUNEL staining was performed with a commercially available kit (ApopTag Plus; Serological Corporation). The numbers of TUNEL-positive cells per field were counted and expressed as the average in 8 fields. To evaluate apoptosis of transplanted MSCs in the ischemic brain, an additional 12 rats (MSC group n=6; MSC+AM group n=6) were euthanized on day 3. Frozen sections were used for TUNEL staining (ApopTag Fluorescein kit). The numbers of TUNEL- and PKH26-positive cells were counted and expressed as the average in 5 sections.

Statistical Analysis

All data were expressed as mean±SEM. Student's unpaired *t* test was used to compare differences between 2 groups. Comparisons of parameters among 4 groups were made by 1-way ANOVA, followed by Newman–Keuls test. Comparisons of the time course of neurological scores were made by 2-way ANOVA for repeated measures, followed by Newman–Keuls test. A P value <0.05 was considered statistically significant.

Results

Engraftment and Differentiation of Transplanted MSCs

Intravenously administered MSCs were engrafted in the ischemic penumbra. Some MSCs were positive for NeuNs and GFAP (Figure 1A and 1B). Other MSCs were positive for

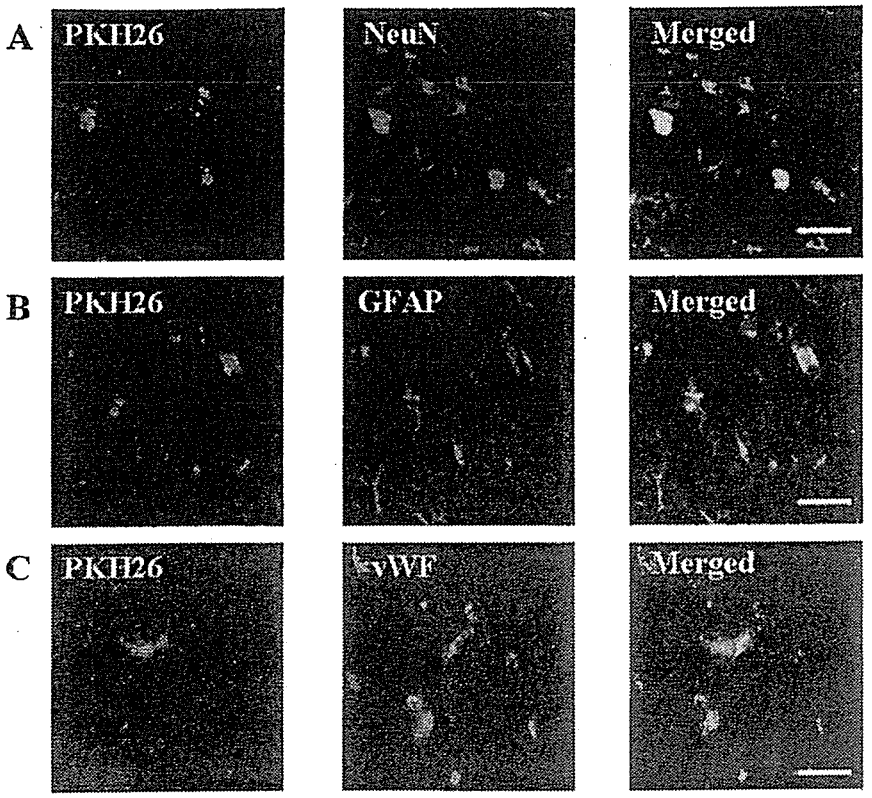

Figure 1. Engraftment and differentiation of transplanted MSCs. PKH26-labeled MSCs were frequently observed in ischemic penumbra. Some PKH26-positive MSCs (red) expressed neuronal marker (NeuN; green; A), astrocyte marker (GFAP; green; B), or endothelial cell marker (vWF; green; C). Bars=20 μm.

vascular endothelial marker vWF (Figure 1C). The numbers of differentiated MSCs did not differ significantly between the MSC and MSC+AM groups (data not shown). Few MSCs were observed in the contralateral nonischemic tissue.

Neurological Assessment

Neurological severity scores on day 1 did not differ significantly among 4 groups (Figure 2). Neurological deficits gradually improved in all groups. Scores in the MSC and AM groups on days 7 and 14 were lower than those in the control group ($P<0.05$), although there were no significant differences between the AM and MSC groups on days 7 and 14. Interestingly, the scores on days 7 and 14 were lowest in the MSC+AM group among the 4 groups.

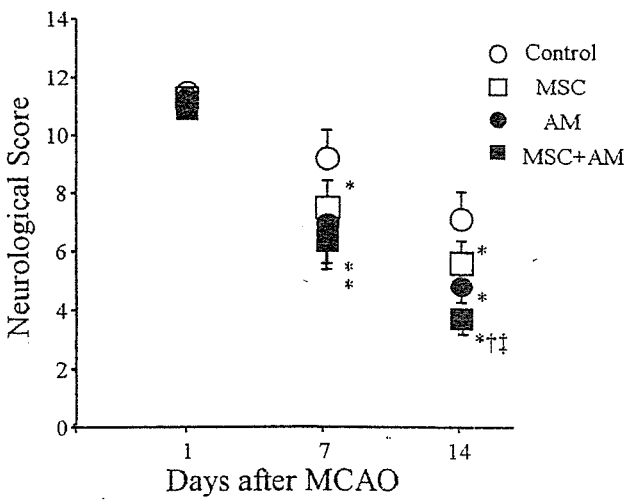

Figure 2. Neurological score on days 1, 7, and 14 in the control group, MSC group, AM group, and MSC+AM group. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs MSC group; ‡$P<0.05$ vs AM group.

Infarct Size and Physiological Data

Infarct size on day 1 in the MSC or AM group was significantly smaller than that in the control group ($P<0.05$; Table 1). Furthermore, the infarct size in the MSC+AM group was the smallest among 4 groups. However, on day 14, there was no significant difference in infarct size, although the infarct size tended to be small in the treatment groups. Percent increase in body weight in the MSC, AM, and MSC+AM groups was higher than that in the control group ($P<0.05$; Table 2).

TABLE 1. Percent Infarct Size to the Contralateral Hemisphere

		Infarct Size (%)	
Group	No.	Day 1	Day 14
Control	8	31±1	31±2
MSC	8	27±1*	29±2
AM	8	28±1*	29±1
MSC+AM	8	25±1*†‡	28±2

Control indicates rats given vehicle infusion; MSC, rats given MSC transplantation; AM, rats given AM infusion; MSC+AM, rats given MSC transplantation and AM infusion.

Data are mean±SEM.

*$P<0.05$ vs control group.

†$P<0.05$ vs MSC group.

‡$P<0.05$ vs AM group.

TABLE 2. Percent Increase of Body Weight

Group	No.	% Increase of Body Weight
Control	16	8±3
MSC	16	12±2*
AM	16	13±2*
MSC+AM	16	14±2*

Control indicates rats given vehicle infusion; MSC, rats given MSC transplantation; AM, rats given AM infusion; MSC+AM, rats given MSC transplantation and AM infusion.

Data are mean±SEM.

*$P<0.05$ vs control group.

Angiogenic Potency of AM and MSCs

Angiogenesis in the ischemic penumbra was observed after MCAO compared with sham operation (Figure 3A). Furthermore, MSC transplantation or AM infusion induced angiogenesis in the ischemic penumbra, and particularly, the angiogenic effect was marked after combined therapy of MSCs and AM. Quantitative analysis demonstrated that the area of vWF staining in the MSC and AM groups was higher than that in the control group ($P<0.05$ versus control group; Figure 3B). There was no significant difference between the MSC and AM groups. Interestingly, the area of vWF staining in the MSC+AM group was highest among the 4 groups ($P<0.05$ versus MSC and AM groups). There were no significant differences in neovascularization of noninfarct tissue in all groups (Figure 3A and 3B). Representative photomicrographs of immunostaining of Ki67, a marker for cell proliferation, demonstrated that AM infusion and MSC transplantation increased the number of Ki67-positive newly formed microvessels in the ischemic penumbra (Figure 3C and 3D).

Antiapoptotic Effects of AM on Neuronal Cells and Transplanted MSCs

TUNEL-positive cells were frequently observed in the ischemic penumbra on day 1 (Figure 4A). Quantitative analysis demonstrated that the number of TUNEL-positive cells in the treatment groups was lower than that in the control group ($P<0.05$ versus control group; Figure 4B). Interestingly, the number of TUNEL-positive cells in the MSC+AM group was significantly lower than that in the MSC and AM groups ($P<0.05$ versus MSC and AM groups), although there was no significant difference between the MSC and AM groups.

The majority of transplanted MSCs were positive for TUNEL staining on day 3 (Figure 5A). Infusion of AM decreased TUNEL-positive MSCs in the ischemic penumbra. Quantitative analysis demonstrated that the number of apoptotic MSCs in the MSC+AM group was significantly lower than that in the MSC group ($P<0.05$; Figure 5B). As a result, the number of engrafted MSCs in the MSC+AM group on day 14 was markedly higher than that in the MSC group ($P<0.05$; Figure 5C). The number of TUNEL-positive non-

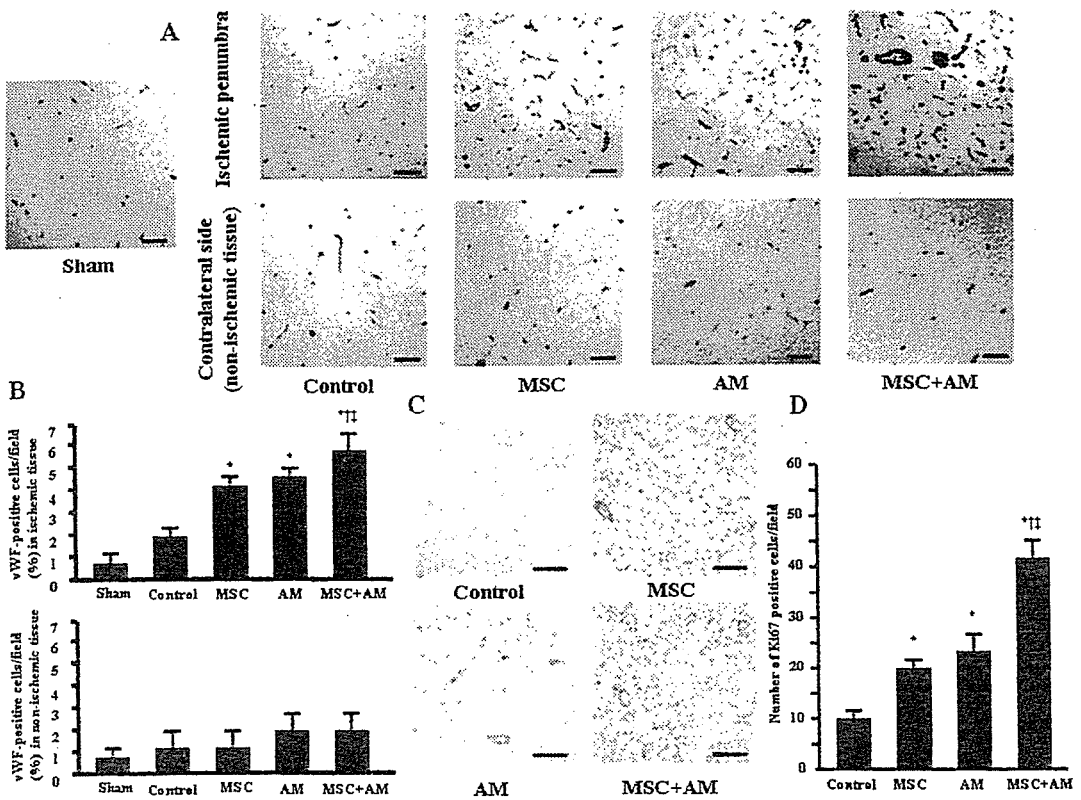

Figure 3. A, Representative photomicrographs of vWF staining in ischemic penumbra (top) and in contralateral nonischemic tissue (bottom). Bars=25 μm. B, Quantitative analysis of angiogenesis using the area of vWF staining in ischemic penumbra (top) and in nonischemic tissue (bottom). C, Representative photomicrographs of Ki67 staining. Bars=50 μm. D, Quantitative analysis of the number of Ki67-positive microvessels. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs MSC group; ‡$P<0.05$ vs AM group.

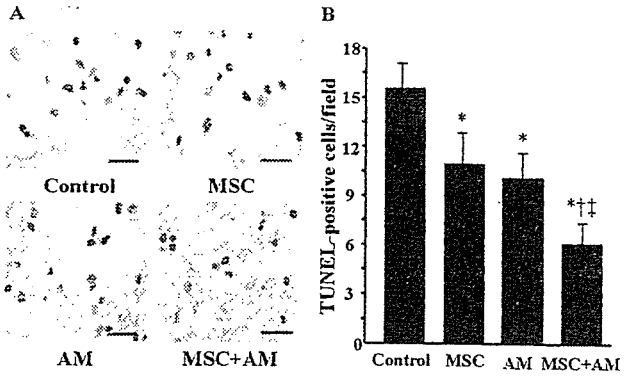

Figure 4. A, Representative photomicrographs of TUNEL staining in ischemic penumbra. The number of TUNEL-positive cells (DAB; brown) in the MSC+AM group was markedly lower than that in the other 3 groups. B, Quantitative analysis of the number of TUNEL-positive cells. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs MSC group; ‡$P<0.05$ vs AM group. Bars=20 μm.

MSCs, including neuronal cells, was also decreased by AM infusion (Figure 5D).

Discussion

In the present study, we demonstrated that: (1) AM infusion or MSC transplantation induced angiogenesis and inhibited apoptosis of neuronal cells in the ischemic penumbra; (2) infusion of AM enhanced the angiogenic potency and antiapoptotic effects of MSC transplantation; (3) AM inhibited apoptosis of transplanted MSCs themselves and increased the number of engrafted MSCs; and (4) combination therapy of AM and MSC induced greater improvement in neurological functions than AM infusion or MSC transplantation alone.

Endogenous AM has been shown to be upregulated by hypoxia in the ischemic brain through a compensatory mechanism.[13] A previous report has demonstrated that pretreatment with AM reduces brain injury and improves neurological deficits in a rat stroke model.[14] The present study demonstrated that AM infusion after the onset of stroke improved neurological functions in rats. However, the underlying mechanisms still remain unclear. We have shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a hindlimb ischemic model via activation of Akt.[6] Expectedly, in the present study, infusion of AM induced neovascularization in the ischemic penumbra. On the other hand, AM has been shown to have potent antiapoptotic effects on various cells through the PI3K/Akt pathway.[7,8] Interestingly, in the present study, short-term infusion of AM markedly decreased TUNEL-positive cells in the ischemic penumbra. AM infusion significantly decreased infarct size on day 1, although the significant change was not observed on day 14. These results suggest that AM improves neurological functions, at least in part, through induction of angiogenesis and inhibition of neuronal cell apoptosis in the ischemic penumbra.

Recently, transplantation of MSCs has been shown to improve neurological functions in experimental stroke.[1,3] The beneficial effects are considered to be mediated by increases in endogenous angiogenic and antiapoptotic factors including VEGF, a potent neuroprotective factor,[12] and by differentiation of MSCs themselves into neuronal cells.[1] The present study showed that MSCs secreted a large amount of VEGF. In fact, we demonstrated in vivo that MSCs induced angiogenesis and inhibited cell apoptosis in the ischemic penumbra (Figures 3 and 4). Furthermore, some transplanted MSCs differentiated into neuronal cells and endothelial cells. Thus, MSCs have neuroprotective effects not only through their differentiation, but also through their ability to secret angiogenic and antiapoptotic factors. Nevertheless, the majority of transplanted MSCs were positive for TUNEL staining on day 3. Interestingly, infusion of AM significantly decreased the

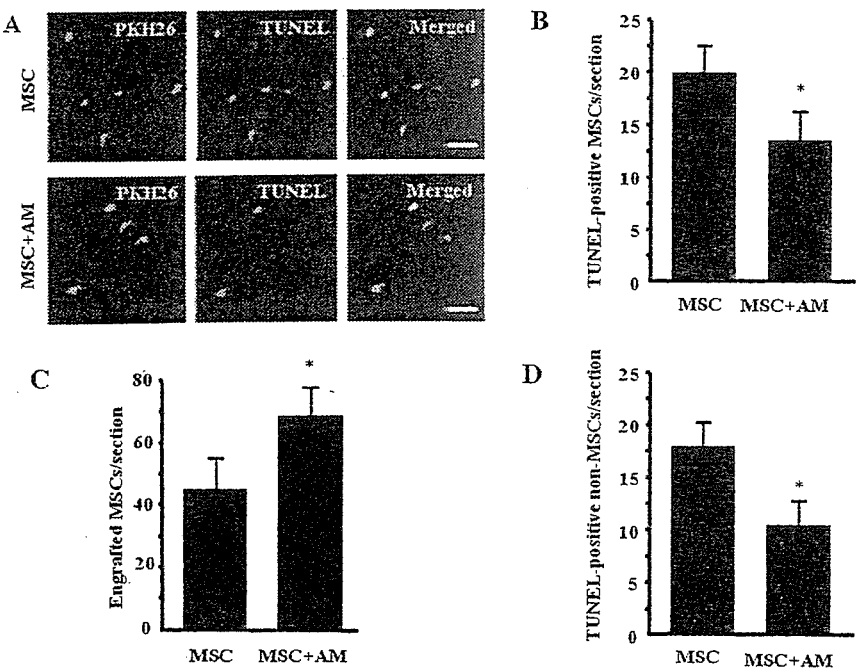

Figure 5. A, Representative photomicrographs of MSC apoptosis after transplantation. Transplanted MSCs were labeled with PKH26. TUNEL-positive cells (green) were frequently observed in ischemic penumbra. Infusion of AM decreased TUNEL-positive MSCs (double-positive cells, merged). B, Quantitative analysis of the number of TUNEL-positive MSCs on day 3. C, The number of engrafted MSCs on day 14. D, Quantitative analysis of the number of TUNEL-positive non-MSCs. Data are mean±SEM. *$P<0.05$. Bars=100 μm.

number of apoptotic cells on day 3. The number of engrafted MSCs in the MSC+AM group on day 14 was markedly higher than that in the MSC group. These results suggest that AM contributes to prolonging the viability of transplanted MSCs. In addition, AM inhibited apoptosis of non-MSCs, suggesting direct protective effects of AM on the ischemic penumbra. Furthermore, a combination of AM infusion and MSC transplantation markedly improved neurological functions compared with MSC transplantation or AM infusion alone. The infarct size on day 1 was smallest in the MSC+AM group, although infarct size on day 14 in the MSC+AM group tended to be small compared with that in other groups. Considering the angiogenic and antiapoptotic effects of AM and MSCs, administered AM may have additional or synergetic effects on MSC transplantation, leading to further improvement in neurological functions after stroke. Interestingly, a significant increase in body weight was observed in rats with low neurological score after treatment. A previous report has shown that body weight after stroke was higher in bFGF-treated rats than in vehicle-treated rats.[15] These results suggest that earlier recovery of neurological deficits might have restored impaired food intake after stroke.

MSC transplantation to treat brain ischemia has been investigated recently. We demonstrated previously the safety of AM infusion in patients with congestive heart failure.[16] Thus, combination therapy using AM infusion and MSC transplantation may be a novel and promising therapeutic strategy for treatment of stroke. However, systemically administered MSCs and AM may develop cancer and retinopathy via their angiogenic potential. Further studies are necessary to examine the safety and efficacy of this treatment.

In conclusion, AM enhanced the therapeutic potency of MSCs, including neurological improvement, possibly through inhibition of MSC apoptosis and induction of angiogenesis. A combination of AM infusion and MSC transplantation may be a new therapeutic strategy for treatment of stroke.

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Effect of cardiopulmonary bypass on pulmonary clearance of adrenomedullin in humans

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Background: Adrenomedullin is a potent vasodilatory peptide and its plasma concentration increases after cardiopulmonary bypass. We analyzed the contribution of the lung to the disposition of adrenomedullin before and after cardiopulmonary bypass in humans.
Methods: Thirty-five patients undergoing cardiac surgery with cardiopulmonary bypass were studied. Bloods were sampled from the pulmonary artery and left atrium at the following times: prior to systemic heparinization, during pulmonary reperfusion and after cardiopulmonary bypass. Plasma concentrations of total and mature adrenomedullin were measured using an immunoradiometric assay kit specific for human adrenomedullin. Intermediate adrenomedullin was calculated as the difference between total adrenomedullin and mature adrenomedullin.
Results: Before cardiopulmonary bypass, mature and intermediate adrenomedullin concentrations were reduced by the pulmonary circulation by approximately 30% and 20%, respectively. However, these effects were not observed during pulmonary reperfusion. Mature, but not intermediate, adrenomedullin was reduced after cardiopulmonary bypass. Furthermore, pulmonary clearance quantity of mature adrenomedullin was significantly enhanced after cardiopulmonary bypass.
Conclusion: These results indicate that cardiopulmonary bypass temporally impairs the pulmonary clearance of mature and intermediate adrenomedullin, but clearance of mature, not intermediate adrenomedullin is enhanced after cardiopulmonary bypass.

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Key words: adrenomedullin; cardiopulmonary bypass; pulmonary clearance.

ADRENOMEDULLIN is a potent vasodilatory peptide isolated from human pheochromcytoma (1). Plasma adrenomedullin concentrations are reported to be increased in patients with cardiovascular diseases, including myocardial infarction, congestive heart failure and pulmonary or systemic hypertension (2–6). We previously demonstrated that plasma adrenomedullin increased during and after cardiopulmonary bypass (CPB) (7). Our subsequent report identified that cerebral adrenomedullin production contributed to the elevated plasma concentration of adrenomedullin after CPB (8). Although the pulmonary circulation is one of the possible sites of augmented adrenomedullin secretion after CPB, there is no evidence that the pulmonary circulation was a source of the increased adrenomedullin (7). On the contrary, two clinical studies documented that the pulmonary circulation may be a site of adrenomedullin clearance (9, 10).

Human adrenomedullin consists of 52 amino acids (1). In the biosynthesis of adrenomedullin, glycine-extended adrenomedullin, an intermediate type adrenomedullin, is produced and then converted to a biologically active or mature adrenomedullin by an amidation enzyme (11). Both mature (active) and intermediate (inactive) adrenomedullin are present in human plasma (11) and increase progressively in proportion to the severity of congestive heart failure (12). The present study was designed to elucidate the role of the pulmonary circulation in the clearance of both types of adrenomedullin before and after CPB in patients undergoing cardiac surgery.

Material and method

The protocol was approved by our institutional human investigation committee and informed consent