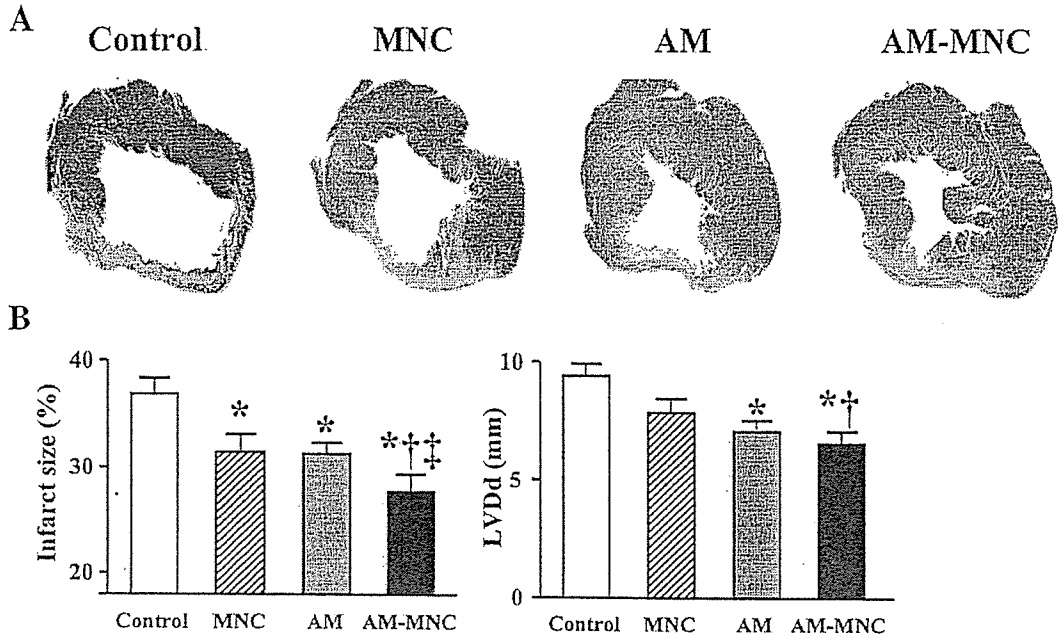

Fig. 1. *A*: representative examples of Masson trichrome-staining of transverse sections of left ventricular (LV) myocardium 4 wk after coronary ligation. *B*: quantitative analysis of infarct size and LV chamber size. Infarcted area and LV end-diastolic diameter (LVDd) of the adrenomedullin-mononuclear 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/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; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ vs. AM.

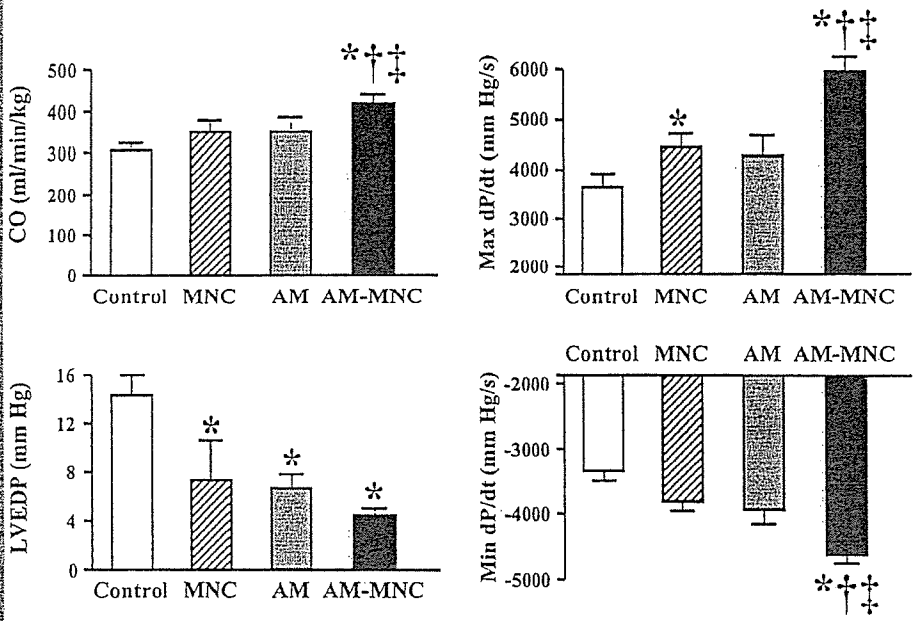

Fig. 2. Effects of AM infusion and MNC transplantation on hemodynamic parameters. CO, cardiac output; LVEDP, LV end-diastolic pressure; Max dP/d*t*, LV maximum change in pressure over time; Min dP/d*t*, LV minimum dP/d*t*. Values are means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. MNC; ‡$P < 0.05$ 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

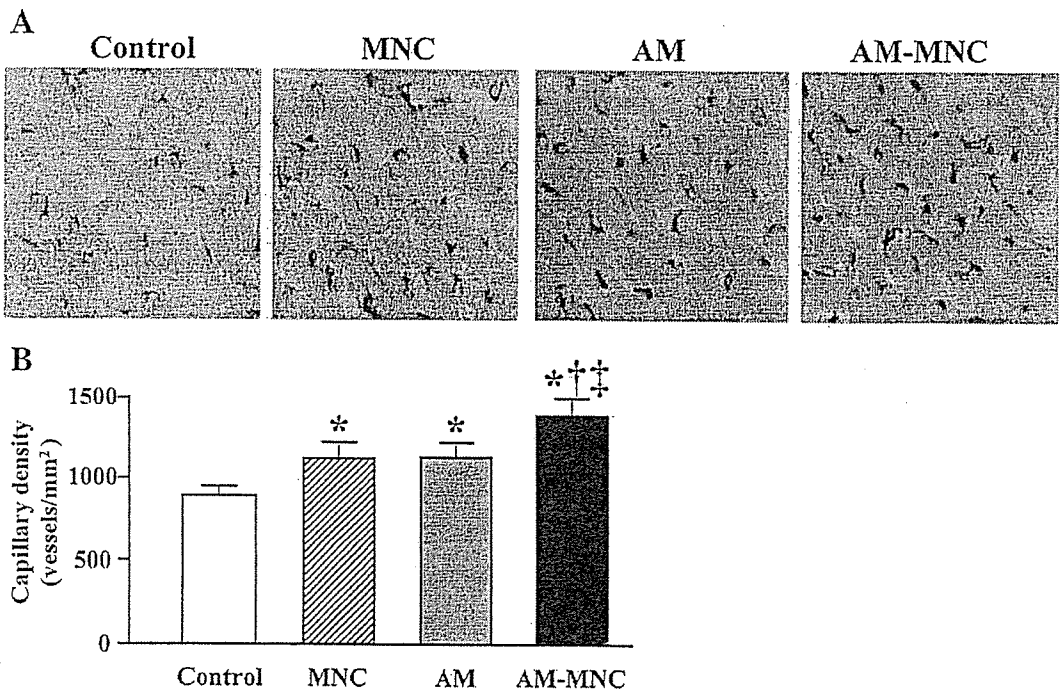

Fig. 3. *A*: representative examples of alkaline phosphatase staining in peri-infarct area. A combination of AM infusion and MNC transplantation markedly induced myocardial neovascularization. Magnification, ×200. *B*: quantitative analysis of capillary density in peri-infarct area. Capillary density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

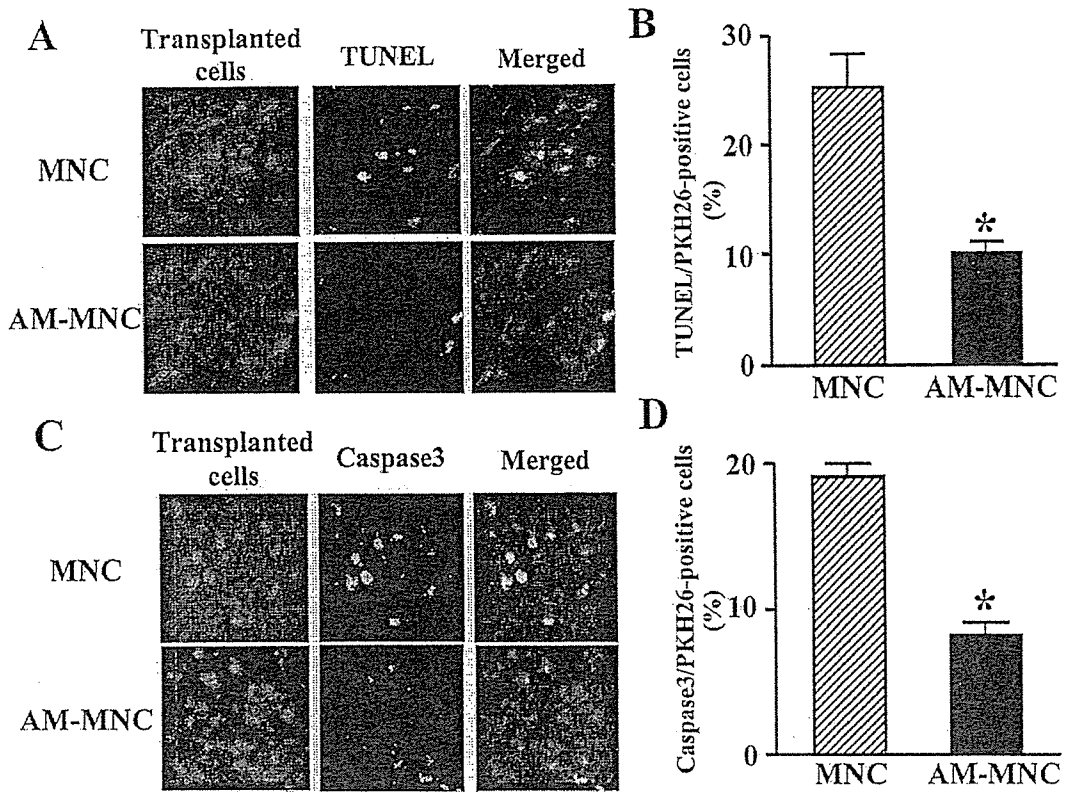

Fig. 4. Detection of transplanted cell apoptosis. *A*: representative photographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Red fluorescence (PKH26) marks transplanted MNC; green fluorescence indicates TUNEL-positive cells. TUNEL-positive cells were frequently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

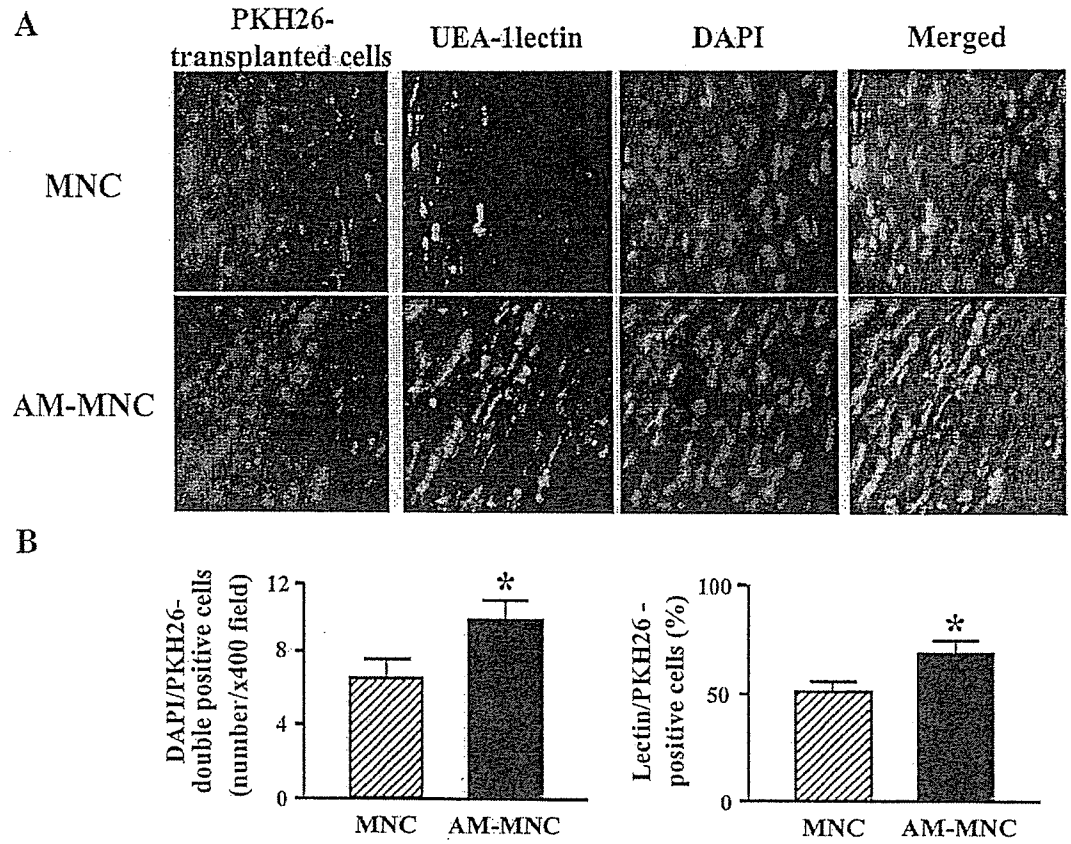

Fig. 5. *A*: representative examples of MNC differentiation into endothelial lineage. Red fluorescence (PKH26) marks transplanted cells; green fluorescence indicates ulex europaeus (UEA)-1 lectin, a marker for vascular endothelial cells. Most of the transplanted cells differentiated into endothelial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

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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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Stroke is available at http://www.strokeaha.org

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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

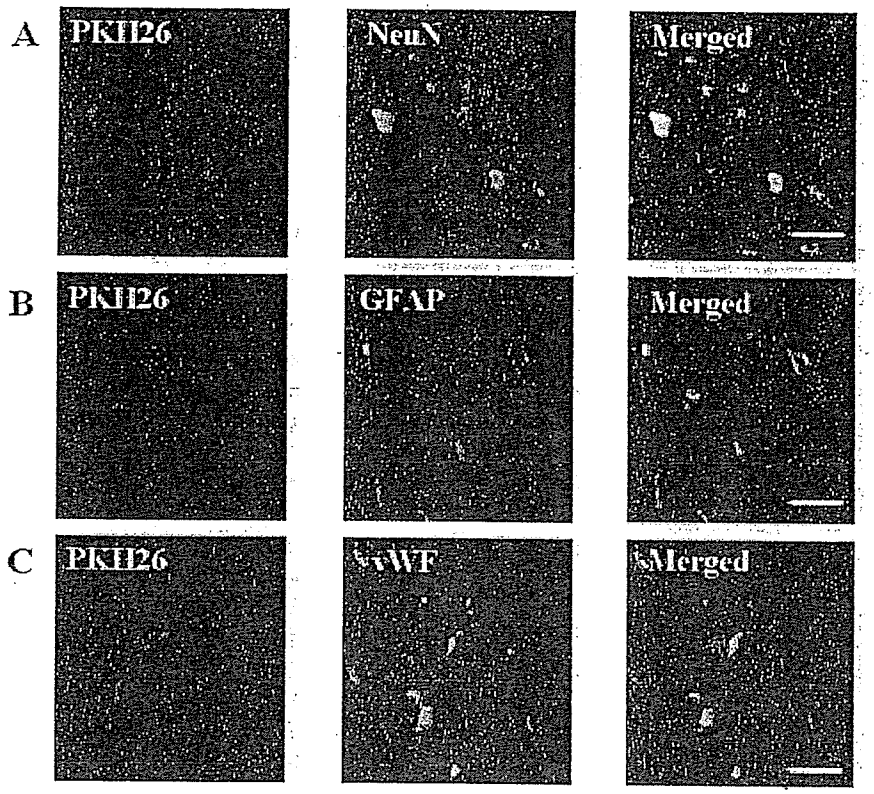

Figure 1. Engraftment and differentiation of transplanted MSCs. PKH26-labeled MSCs were frequently observed in ischemic penumbra. Some PKH26-positive MSCs (red) expressed neuronal marker (NeuN; green; A), astrocyte marker (GFAP; green; B), or endothelial cell marker (vWF; green; C). Bars=20 μm.

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.

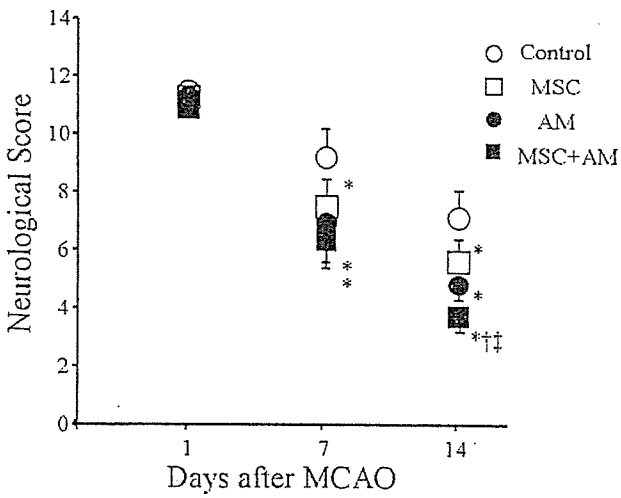

Figure 2. Neurological score on days 1, 7, and 14 in the control group, MSC group, AM group, and MSC+AM group. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs MSC group; ‡$P<0.05$ vs AM group.

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-

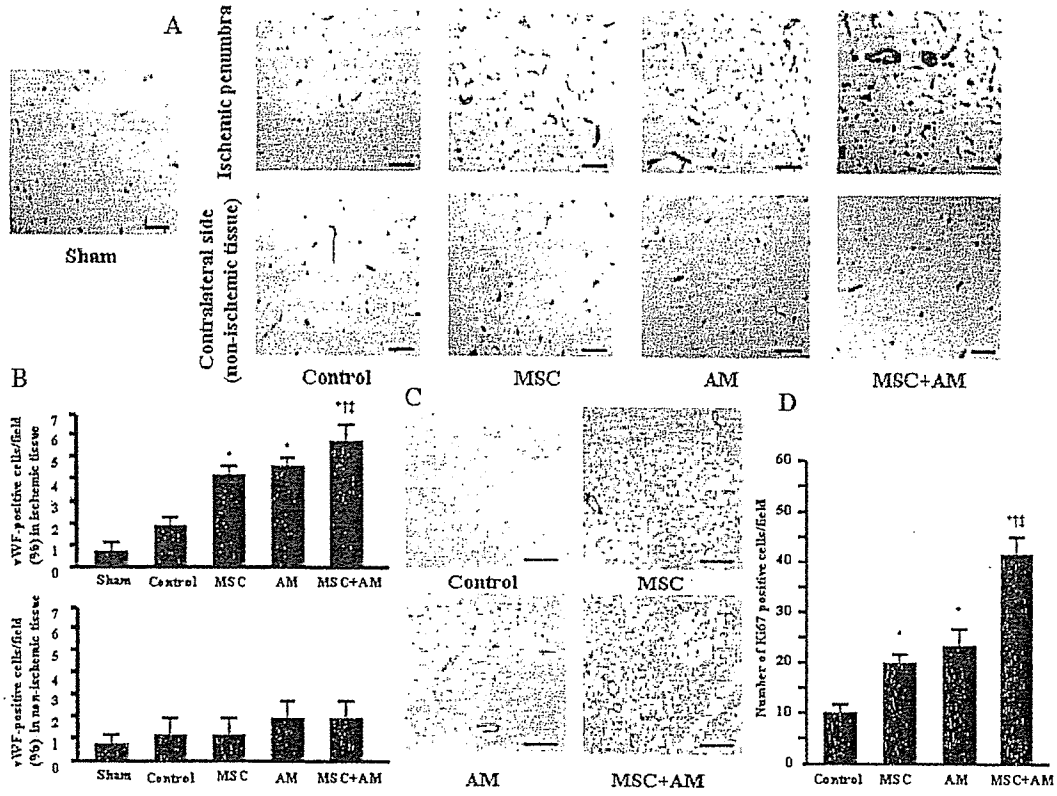

Figure 3. A, Representative photomicrographs of vWF staining in ischemic penumbra (top) and in contralateral nonischemic tissue (bottom). Bars=25 μm. B, Quantitative analysis of angiogenesis using the area of vWF staining in ischemic penumbra (top) and in nonischemic tissue (bottom). C, Representative 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.

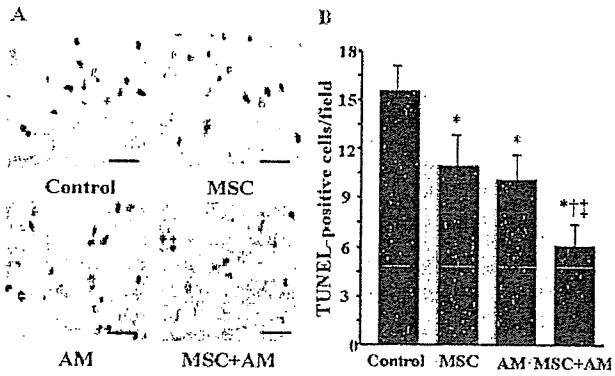

Figure 4. A, Representative photomicrographs of TUNEL staining in ischemic penumbra. The number of TUNEL-positive cells (DAB; brown) in the MSC+AM group was markedly lower than that in the other 3 groups. B, Quantitative analysis of the number of TUNEL-positive cells. Data are mean±SEM. *P<0.05 vs control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

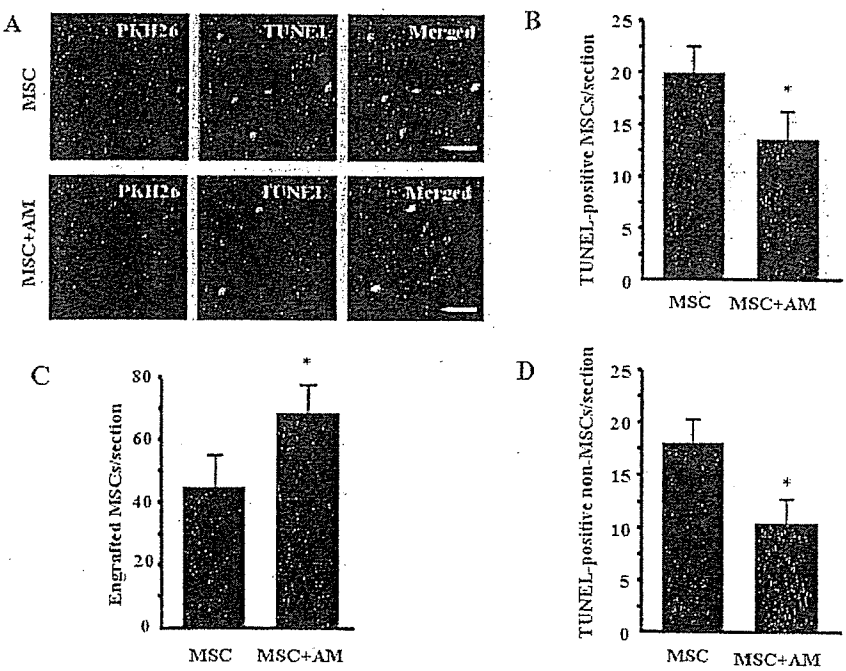

Figure 5. A, Representative photomicrographs of MSC apoptosis after transplantation. Transplanted MSCs were labeled with PKH26. TUNEL-positive cells (green) were frequently observed in ischemic penumbra. Infusion of AM decreased TUNEL-positive MSCs (double-positive cells; merged). B, Quantitative analysis 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.

Acknowledgments

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Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia

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Abstract

Objective: Mesenchymal stem cells (MSC) are pluripotent cells that differentiate into a variety of cells including endothelial cells and vascular smooth muscle cells. Although transplantation of bone marrow-derived mononuclear cells (MNC) has already been applied for the treatment of critical limb ischemia, little information is available regarding comparison of the angiogenic potency between MSC and MNC. Accordingly, we injected equal numbers of MSC or MNC in a rat model of hindlimb ischemia and compared their therapeutic potential.
Methods and results: Immediately after creating hindlimb ischemia, rats were randomized to receive MSC transplantation (MSC group), MNC transplantation (MNC group), or vehicle infusion (Control group). Three weeks after transplantation, the laser Doppler perfusion index was significantly higher in the MNC group than in the Control group (0.69 ± 0.1 vs. 0.57 ± 0.06, $P<0.01$). Furthermore, there was a marked improvement in blood perfusion in the MSC group (0.81 ± 0.08). Capillary density was highest in the MSC group. The number of transplanted cell-derived endothelial cells was higher in the MSC group than in the MNC group. Transplanted cell-derived vascular smooth muscle cells were detected only in the MSC group. In vitro, MSC were more tolerant to apoptotic stimulus (serum starvation and hypoxia) than MNC.
Conclusions: MSC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation. Compared with MNC, MSC survived well under an ischemic environment, and differentiated into not only endothelial cells but also vascular smooth muscle cells. Thus, MSC transplantation may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

Keywords: Angiogenesis; Apoptosis; Cell therapy

1. Introduction

Peripheral vascular disease is a major health care problem in an aging society [1]. In the late stages of peripheral vascular disease, progression of tissue hypoperfusion results in ischemic ulceration and gangrene. Unfortunately, amputation is required in more than a third of these patients. Transplantation of bone marrow-derived mononuclear cells (MNC) has been shown to induce therapeutic neovascularization in critical limb ischemia [2–4]. Thus, MNC have been established as a tool for cell therapy. However, MNC transplantation requires harvesting a large

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amount of bone marrow under general anesthesia, which may impose a load on some patients with severe complications such as myocardial ischemia, heart failure, cerebral disease, or renal failure.

Mesenchymal stem cells (MSC), which reside in the bone marrow stroma, possess pluripotency and differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells, endothelial cells and vascular smooth muscle cells [5–7]. MSC have an adherent nature and are expandable in culture. Thus, it would be easy to obtain a sufficient number of MSC for cell therapy. MSC have been shown to form capillary-like structures in an in vitro Matrigel assay [8]. Furthermore, MSC transplantation has been shown to induce neovascularization in a rat model of hindlimb ischemia [9]. These findings suggest that, like MNC, transplantation of MSC may have beneficial effects in patients with critical limb ischemia. However, there has been no study on the therapeutic potency of MSC compared with that of MNC.

Thus, the purposes of this study were (1) to compare the therapeutic potencies of MSC and MNC transplantation in a rat model of hindlimb ischemia and (2) to investigate the mechanisms underlying the angiogenic potential of MSC.

2. Methods

2.1. Animal model of hindlimb ischemia

Male Lewis rats weighing 250 to 275 g were used in this study. These isogenic rats served as donors and recipients to simulate autologous implantation of MSC or MNC. To create a hindlimb ischemia model, the left common iliac artery of each rat was resected under anesthesia with pentobarbital sodium (50 mg/kg i.p.). The distal portion of the saphenous artery and all side branches as well as veins were dissected free and excised. The right hindlimb was kept intact and used as a nonischemic limb. All protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Expansion of MSC and isolation of MNC

MSC expansion was performed as described previously [7]. In brief, we sacrificed male Lewis rats and harvested bone marrow by flushing the cavity of the femurs and tibias with phosphate-buffered saline (PBS). Bone marrow cells were introduced into 100-mm dishes and cultured in α-MEM supplemented with 10% FBS and antibiotics. A small number of cells developed visible symmetric colonies by days 5 to 7. Nonadherent hematopoietic cells were removed and the medium was replaced. The adherent, spindle-shaped MSC population expanded to over 50 million cells at approximately 4 to 5 passages after first culturing the cells. MNC were isolated from whole marrow cells by Ficoll density gradient centrifugation (Lymphoprep, Nycomed) as described previously [3].

2.3. MSC and MNC transplantation

Immediately after resection of the left common iliac artery, 30 rats were randomized to the following three groups: (1) MSC transplantation (MSC group, n=10), (2) MNC transplantation (MNC group, n=10), and (3) PBS injection (Control group, n=10). In each group, 5×10^6 MSC, 5×10^6 MNC or PBS was injected into the ischemic thigh muscle with a 26-gauge needle at five different points.

2.4. Assessment of blood perfusion

A laser Doppler perfusion image (LDPI) analyzer (Moor Instruments) was used to measure serial blood flow over a period of 3 weeks. Low or no blood perfusion was displayed as dark blue, whereas the highest perfusion was displayed as red. After blood flow had been scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification using stored images. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion [10].

Blood flow of the ischemic hindlimb was also assessed by use of colored microspheres 3 weeks after transplantation as reported previously [11,12]. In brief, 2×10^6 microspheres (Dye-Trak, Triton Technology) were injected into the left ventricle under anesthesia and artificial ventilation. Reference blood samples were withdrawn from the right carotid artery at a rate of 0.3 ml/min. Rats were then killed, and muscle samples from the adductor, semimembranous and gastrocnemius muscles were harvested and weighed. These samples were processed according to the manufacturer's instructions, and the absorbance of dye from muscle samples (AU_{muscle}) and reference blood samples (AU_{sample}) was measured with a spectrophotometer. The regional blood flow of ischemic muscle was calculated as $0.3\times(AU_{muscle})/(AU_{sample})$ and presented normalized per 100 g body weight.

2.5. Histological assessment

Rats were killed 3 weeks after transplantation. Four 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 using an indoxyltetrazolium method to detect capillary endothelial cells [3,10]. Five fields from four tissue sections were randomly selected, and the number of capillaries was counted in each field. To avoid overestimation or underestimation of capillary density as a result of myocyte atrophy or interstitial edema, the

capillary number adjusted per muscle fiber was used to compare the differences in capillary density among the three groups [3].

2.6. Monitoring of transplanted MSC or MNC in ischemic hindlimb muscle

An additional 10 rats were used to examine whether transplanted MSC or MNC differentiate into endothelial cells or vascular smooth muscle cells in ischemic muscle. Suspended MSC and MNC were labeled with fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit, Sigma Chemical) as reported previously [13]. Red fluorescence-labeled MSC (5×10^6 cells per animal) or MNC (5×10^6 cells per animal) were transplanted into the ischemic thigh muscle in rats (MSC and MNC groups, n=5 each). This subgroup of rats was killed 3 weeks after MSC or MNC transplantation, and frozen tissue sections were obtained from the ischemic muscle. The tissue sections were incubated with anti-von Willebrand factor polyclonal antibody (vWF, DAKO) or anti-α-smooth muscle actin monoclonal antibody (αSMA, DAKO), followed by incubation with Alexa Fluor® 633 IgG antibody (Molecular Probes) or fluorescein isothiocynate (FITC)-conjugated IgG antibody (BD Pharmingen), respectively. Five high power fields (40×) of each section were randomly selected to count the number of transplanted cells, vWF-positive cells, and αSMA-positive cells.

Green fluorescent protein (GFP)-expressing MSC or MNC were also transplanted to examine cell differentiation. MSC and MNC were isolated from male GFP-transgenic rats, which were provided by Dr. Masaru Okabe (Osaka University, Japan) [14,15]. GFP-expressing MSC (5×10^6 cells per animal) or MNC (5×10^6 cells per animal) were transplanted into the ischemic thigh muscle in male Spargue–Dawley rats (MSC group, n=3; MNC group, n=3). Immunohistochemical analysis for vWF was performed 3 weeks after cell transplantation.

2.7. Cell viability assay

MSC or MNC were plated in serum-free medium on 12-well plates (1×10^5 cells per well) and cultured in hypoxic conditions (1% O_2/5% CO_2/94% N_2) for 24 h.

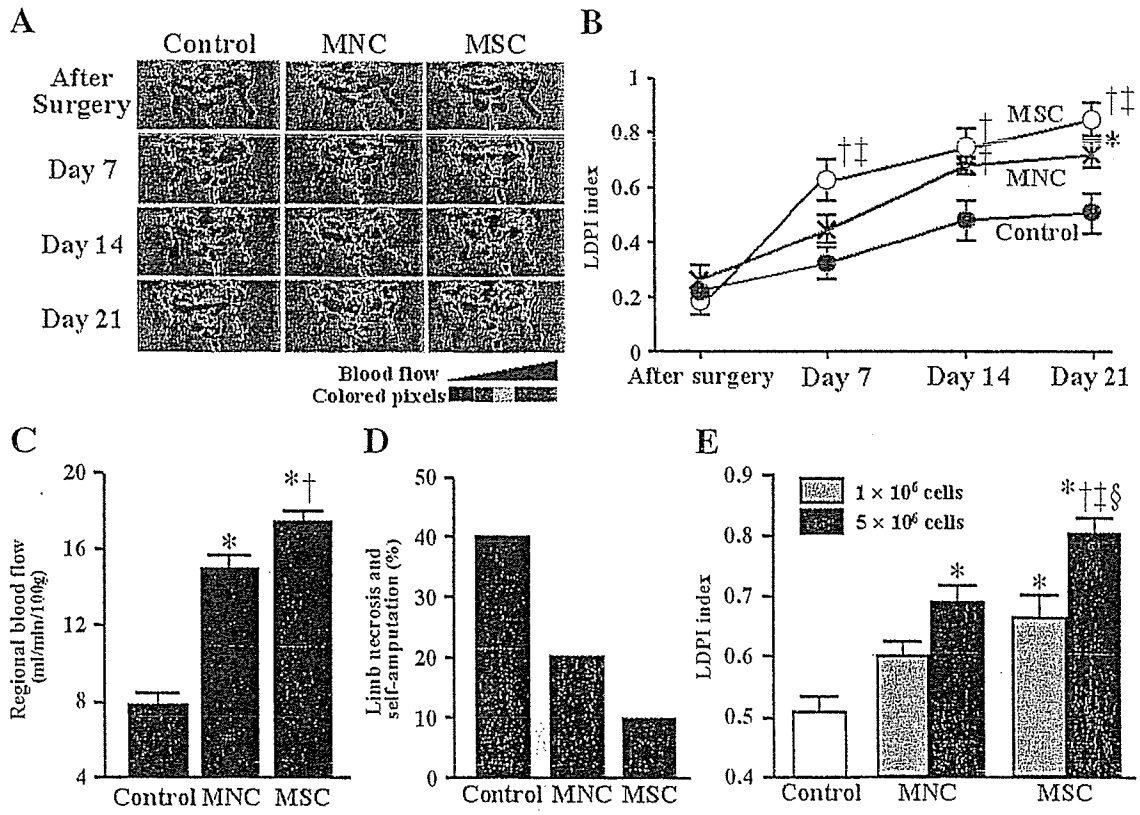

Fig. 1. (A) Representative examples of serial LDPI. Blood perfusion of the ischemic hindlimb markedly increased in the MSC group 3 weeks after transplantation (red to orange). (B) Quantitative analysis of hindlimb blood perfusion. LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion. The increase in the LDPI index was not significant in the Control group. LDPI index was significantly higher in the MNC and MSC groups than in the Control group 3 weeks after surgery. LDPI index in the MSC group was highest among the three groups. Data are mean±S.E.M. *P<0.05 and †P<0.01 vs. Control; ‡P<0.01 vs. MNC. (C) Blood flow assessment of ischemic muscle by use of microspheres. Data are mean±S.E.M. *P<0.01 vs. Control; †P<0.05 vs. MNC. (D) Incidence of limb necrosis and self-amputation 3 weeks after transplantation. (E) Comparison between the number of transplanted cells and perfusion recovery of the ischemic hindlimb. LDPI index of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation. Data are mean±S.E.M. *P<0.01 vs. Control; †P<0.01 vs. 1×10^6 MNC; ‡P<0.05 vs. 5×10^6 MNC; §P<0.05 vs. 1×10^6 MSC.

Hypoxic conditions were obtained by the use of a CO_2/multigas incubator (ASTEC). After exposure to serum-free hypoxia, the cells were suspended using trypsin and mixed with 0.4% trypan blue solution (Sigma Chemical). Percentages of viable cells were evaluated by two blinded observers and normalized to the total cell number in the field.

2.8. In vitro apoptosis assay

Terminal dUTP nick-end labeling (TUNEL) assay (ApopTag Fluorescein kit, Serological Corporation) was performed to evaluate apoptosis of MSC or MNC induced by the combination of serum starvation and hypoxia. After incubation for 24 h, MSC or MNC were fixed in 1% paraformaldehyde, and TUNEL was performed for detection of apoptotic nuclei according to the manufacturer's instructions. The cells were then mounted in medium containing 4′,6-diamidino-2-phenylindole (DAPI). Randomly selected microscopic fields (n=5) were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

2.9. Western blot analysis of hypoxia-inducible factor (HIF)

To identify the protein expression of HIF-1α, Western blotting was performed using a mouse monoclonal antibody raised against HIF-1α (clone H1α67, Novus Biologicals). Serum-starved MSC or MNC were cultured in normoxic or hypoxic conditions for 24 h. Cells were homogenized on ice in 150 μl lysis buffer with a protease inhibitor. Then 15 μg of protein was transferred to sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane. After being blocked for 60 min, the membrane was incubated with a primary antibody in blocking buffer (1:1000), followed by incubation with a peroxidase-conjugated secondary antibody. Positive protein bands were visualized by chemiluminesence using an ECL kit (Amersham). Western blot analysis using a monoclonal antibody raised against β-actin (Sigma Chemical) was used as a protein loading control.

2.10. In vitro tube formation assay

In vitro Matrigel assay was performed to investigate whether MSC or MNC induce tube formation under the condition of serum-free hypoxia [6,8]. MSC or MNC were plated in serum-free medium on 12-well plates (1×10^6 cells per well) coated with Matrigel (Becton Dickinson). After 6-h incubation in hypoxia, tube formation was examined with a phase-contrast microscope.

2.11. Measurements of angiogenic factors

To compare the secretion of angiogenic factors from MSC with that from MNC, a total of 1×10^6 MSC or MNC were plated in serum-free medium on 6-well plates. After 24-h incubation, the conditioned medium was

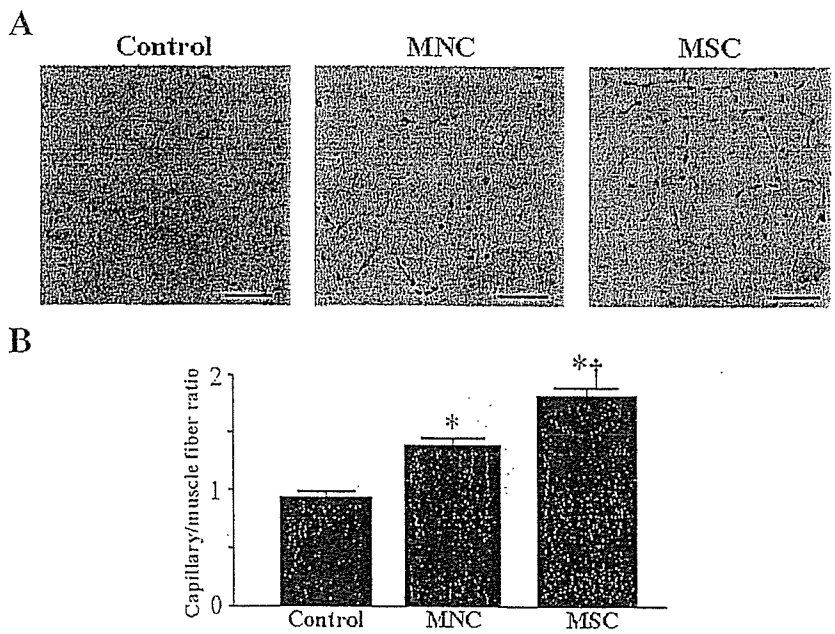

Fig. 2. (A) Representative examples of alkaline phosphatase staining in ischemic hindlimb muscles. In the MNC and MSC groups, the number of capillaries markedly increased compared with the Control group. (B) Quantitative analysis of capillary density in ischemic hindlimb muscles. Capillary density is shown as capillary/muscle fiber ratio. The capillary/muscle fiber ratio of ischemic hindlimb muscle was highest in the MSC group, followed by the MNC group and Control group. Data are mean±S.E.M. *P<0.01 vs. Control; †P<0.01 vs. MNC. Scale Bars: 50 μm.

collected and, levels of VEGF, basic FGF (bFGF), and stromal cell-derived factor-1α (SDF-1α) were measured using enzyme immunoassay kits (Quantikine, R&D Systems).

2.12. Statistical analysis

All values are expressed as mean±S.E.M. Comparisons of parameters among the three groups were made by one-way ANOVA followed by Scheffe's multiple comparison test. Student's unpaired *t*-test was used to compare differences between two groups. A probability value <0.05 was considered statistically significant.

3. Results

3.1. Blood perfusion of ischemic hindlimb after transplantation

Blood perfusion of the ischemic hindlimb was considerably impaired 3 weeks after surgery (Control group, Fig. 1A). On the other hand, improvement of hindlimb ischemia was observed in the MNC and MSC groups compared with the Control group. Hindlimb ischemia was markedly improved in the MSC group. Quantitative analysis demonstrated that the LDPI index was highest in the MSC group, followed by the MNC group and

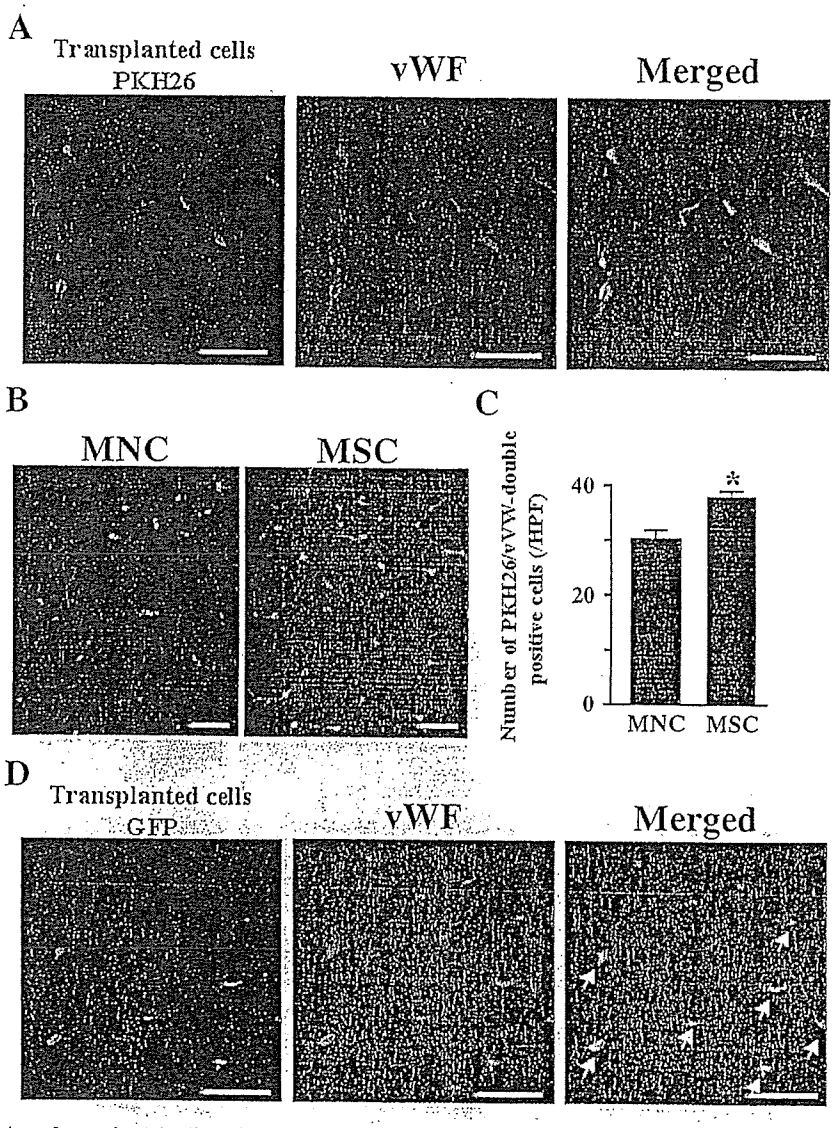

Fig. 3. Endothelial differentiation of transplanted cells. (A) Incorporation and differentiation of transplanted MSC in ischemic thigh muscle. Red fluorescence (PKH26)-labeled MSC were incorporated into interstitial tissues between muscle fibers and were positive for vWF (blue). (B) PKH26/vWF-double-positive cells (pink) were frequently observed in the MSC group. (C) The number of PKH26/vWF-double-positive cells (MNC-derived endothelial cells) was significantly higher in the MSC group than in the MNC group. Data are mean±S.E.M. *$P<0.01$ vs. MNC. Bars: 50 μm. (D) Representative photographs of MSC-derived vascular structures. Green fluorescent protein (GFP)-expressing MSC were transplanted into ischemic thigh muscle. GFP (green)/vWF (red)-double positive cells (orange, arrows) were observed 3 weeks after transplantation. Bars: 50 μm.

Control group (Fig. 1B). The regional blood flow of ischemic muscle was highest in the MSC group, followed by the MNC group and Control group (Fig. 1C). The incidence of limb necrosis and self-amputation of ischemic hindlimb was minimal in the MSC group, followed by the MNC group and Control group (Fig. 1D).

To examine the relationship between the number of transplanted cells and their angiogenic potential, a smaller number of MSC or MNC (1×10^6 cells per animal) was transplanted into the ischemic thigh muscle (n=10 each). Transplantation of 1×10^6 MNC tended to improve blood perfusion of the ischemic hindlimb 3 weeks after transplantation, but these changes did not reach statistical significance. On the other hand, transplantation of 1×10^6 MSC significantly improved hindlimb ischemia. The LDPI index of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation (Fig. 1E).

3.2. Capillary density of ischemic hindlimb

A large number of capillaries were detected in the ischemic muscle of the MSC and MNC groups (Fig. 2A). Importantly, quantitative analysis demonstrated that the capillary/muscle fiber ratio of ischemic muscle was highest in the MSC group, followed by the MNC group and Control group (Fig. 2B).

3.3. Endothelial differentiation of transplanted cells

Red fluorescence-labeled MSC and MNC were detected in the interstitial tissues between muscle fibers 3 weeks after transplantation (Fig. 3A). PKH26-positive cells expressed vWF, an endothelial marker, in both the MSC and MNC groups. Importantly, PKH26/vWF-double-positive cells were frequently observed in the MSC group (Fig. 3B). Quantitative analysis demonstrated that the number of PKH26/vWF-double-positive cells was significantly higher in the MSC group than in the MNC group (Fig. 3C). GFP-expressing MSC and MNC were also detected in the ischemic muscle and were positive for vWF 3 weeks after transplantation (Fig. 3D). The number of GFP/vWF-double-positive cells was significantly higher in the MSC group than in the MNC group (data not shown).

3.4. Differentiation of transplanted cells into vascular smooth muscle cells

Some of the transplanted MSC were positive for αSMA, a marker of vascular smooth muscle cells. They participated in the formation of vascular structures as mural cells. In contrast, none of the MNC was stained by αSMA (Fig. 4).

3.5. Cell survival under serum starvation and hypoxia

After 24-h incubation under serum-free and hypoxic conditions, the majority of cultured MNC revealed cytoplasmic shrinkage, disintegration into small vesicles, and membrane blebbing (Fig. 5A). In contrast, these morphological changes were rarely observed in MSC. The percentage of dead cells, assessed by the trypan blue exclusion test, was significantly higher in MNC than in MSC (Fig. 5B). TUNEL staining showed that serum-free hypoxia markedly induced MNC apoptosis, whereas only a small number of MSC were TUNEL-positive (Fig. 5C). The

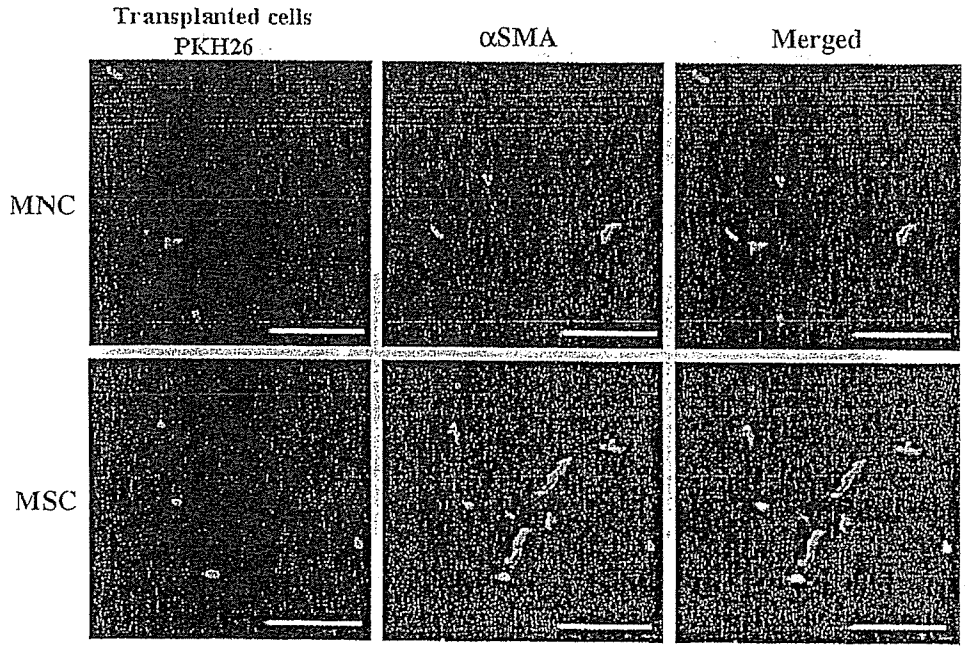

Fig. 4. Differentiation of transplanted cells into vascular smooth muscle cells. Red fluorescence (PKH26)-labeled MSC were stained by αSMA, whereas transplanted MNC did not express αSMA. Bars: 50 μm.

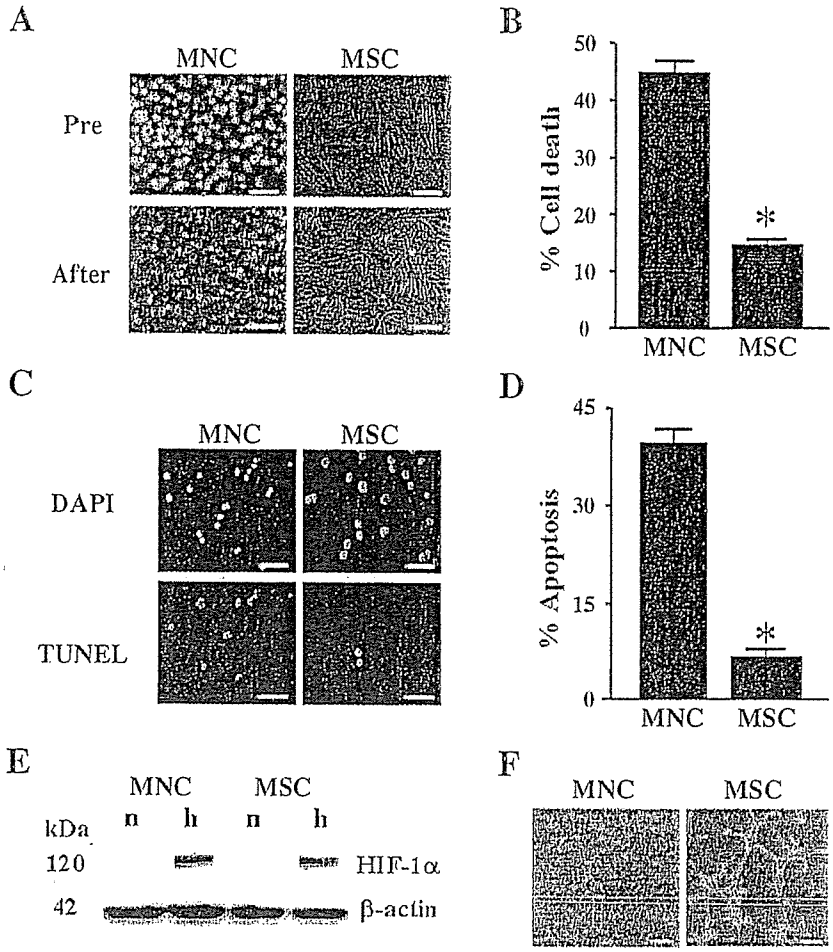

Fig. 5. (A–D) In vitro cell viability assay and apoptosis assay. (A) Representative photographs of MSC and MNC. (B) Quantitative analysis of cell viability by trypan blue staining. The percentage of dead cells in MSC was significantly lower than that in MNC. (C) Representative photographs of apoptotic MSC and MNC. Apoptosis of MSC or MNC was detected by TUNEL staining (green). Nuclei were stained with DAPI (blue). Serum starvation and hypoxia substantially induced MNC apoptosis. (D) Quantitative analysis of TUNEL-positive cells. The percentage of TUNEL-positive cells in MNC was significantly higher than that in MSC. Data are mean±S.E.M. *P<0.01 vs. Control. (E) Western blot analysis for hypoxia-inducible factor (HIF)-1α and β-actin. The expression of HIF-1α protein was not detected in MSC and MNC under the condition of normoxia (n). However, HIF-1α protein was expressed in both cell types after exposure to serum-free hypoxia (h). (F) Representative photographs of in vitro Matrigel assay. After 6-h incubation in serum-free hypoxia, MSC formed typical tube-like structures. In contrast, MNC did not show any morphological change. Bars: 100 μm.

percentage of TUNEL-positive cells was significantly higher in MNC than in MSC (Fig. 5D). The expression of HIF-1α protein was observed in both MSC and MNC under serum-free and hypoxic conditions (Fig. 5E). The ratios of HIF-1α/β-actin did not significantly differ between MSC and MNC (data not shown).

3.6. Tube formation under serum starvation and hypoxia

After 6-h incubation on Matrigel, tube formation was observed in MSC, whereas MNC did not show any morphological change (Fig. 5F).

3.7. Secretion of angiogenic factors from MSC and MNC

VEGF and bFGF were detected in conditioned medium of cultured MSC and MNC. Compared with MNC, MSC secreted significantly greater amounts of VEGF and bFGF (VEGF: 817±36 vs. 188±32 pg/10^6 cells, P<0.01; bFGF: 47±5 vs. 4±1 pg/10^6 cells, P<0.01). Although SDF-1α was not detected in conditioned medium of MNC, MSC secreted a large amount of SDF-1α (17±1 ng/10^6 cells).

4. Discussion

In the present study, we demonstrated that (1) transplantation of MSC as well as MNC induced angiogenesis in a rat model of hindlimb ischemia, (2) the extent of neovascularization was significantly greater in MSC transplantation than in MNC transplantation, (3) transplanted MSC highly differentiated into endothelial cells compared with transplanted MNC, and (4) only MSC differentiated into vascular smooth muscle cells in ischemic tissue. We also demonstrated in vitro that (5) MSC were more tolerant to an ischemic stimulus than

MNC and that (6) MSC secreted large amounts of angiogenic factors compared with the amounts secreted by MNC.

Earlier studies have shown that MNC transplantation enhances neovascularization by supplying endothelial progenitor cells and multiple angiogenic factors such as VEGF, bFGF, and angiopoietin-1 [3,4,16]. In fact, MNC transplantation significantly augmented blood perfusion and capillary density in the ischemic hindlimb in the present study. Other studies have shown that transplanted MSC differentiate into endothelial cells, secrete angiogenic factors, and thereby induce neovascularization in ischemic tissue [9,17,18]. However, it remains unclear whether the angiogenic potency of MSC transplantation is comparable or superior to that of MNC transplantation. In the present study, we injected equal numbers of MSC or MNC into ischemic muscle to compare the therapeutic effects of the two types of cells. Interestingly, MSC transplantation markedly increased blood perfusion and capillary density in the ischemic hindlimb compared with MNC transplantation. Moreover, perfusion recovery of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation. These results suggest that MSC transplantation is more potent in therapeutic angiogenesis than MNC transplantation.

The underlying mechanisms responsible for the superiority of MSC in therapeutic angiogenesis remain unknown. Earlier studies have shown that many transplanted cells undergo apoptosis immediately after transplantation because of a lack of oxygen and nutrition, although they should survive for a sufficiently long period to induce angiogenesis [3,19]. In fact, the present study showed in vitro that MNC readily underwent cell death and apoptosis under conditions of serum starvation and hypoxia. These findings raise the possibility that the therapeutic potency of transplanted MNC is considerably attenuated by an ischemic environment. In contrast, MSC survived well under these conditions. Thus, MSC may be more appropriate for cell transplantation with respect to cell survival than MNC.

The present study showed that transplanted MSC and MNC participated in vascular structures and expressed vWF, an endothelial cell marker. The number of MSC-derived vWF-positive cells in ischemic muscle was significantly higher than that of MNC-derived vWF-positive cells. Previous studies have shown that both transplanted MSC and MNC are capable of differentiating into endothelial cells in ischemic tissue [3,9]. However, the present study showed that a combination of serum starvation and hypoxia greatly reduced MNC viability. Furthermore, only MSC induced tube formation in serum-free and hypoxic conditions. Taking these results together, it is interesting to speculate that transplanted MSC survive well and differentiate into endothelial cells in an ischemic environment and thereby induce angiogenesis more efficiently than transplanted MNC.

During the process of neovascularization, vascular smooth muscle cells play an important role in vessel maturation [20,21]. In the present study, none of the transplanted MNC expressed αSMA, which is consistent with recent findings that MNC-derived CD34-positive cells rarely expressed a vascular smooth muscle cell marker and highly differentiated into endothelial cells in ischemic muscle [22]. On the other hand, earlier studies have shown that MSC readily acquire vascular smooth muscle properties in vitro and that transplanted MSC differentiate into vascular smooth muscle cells in ischemic tissue [9,23]. The present study also demonstrated that some transplanted MSC were positive for αSMA, a vascular smooth muscle cell marker, and formed vascular structures as mural cells. Thus, unlike MNC, transplanted MSC may contribute to vessel maturation.

Recent studies have demonstrated that the angiogenic potential of MSC and MNC is attributed not only to their differentiation into vascular endothelial cells but also to their ability to produce various angiogenic factors, including VEGF and bFGF [4,16–18]. The present study demonstrated that MSC secreted large amounts of VEGF and bFGF compared with the amounts secreted by MNC. Interestingly, only MSC significantly secreted SDF-1α, which also has been shown to induce angiogenesis in vivo and in vitro [24,25]. These findings suggest that MSC transplantation induces angiogenesis more efficiently than MNC transplantation partly through the release of angiogenic factors.

From a clinical standpoint, MNC transplantation is considered to be an established procedure that is easy to implement without any immunosuppressive agents and expensive facilities [2,4,26–31]. In contrast, MSC transplantation requires time and considerable cost to obtain an adequate number of MSC under strictly aseptic conditions. Nevertheless, MSC are an attractive source for cell therapy because they are easily isolated from a small amount of bone marrow and rapidly expand in culture. Thus, MSC transplantation may be one of the most attractive cell therapies in the treatment of critical limb ischemia.

In conclusion, MSC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation. Compared with MNC, MSC survived well in an ischemic environment and differentiated into not only endothelial cells but also vascular smooth muscle cells. Thus, MSC transplantation may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

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