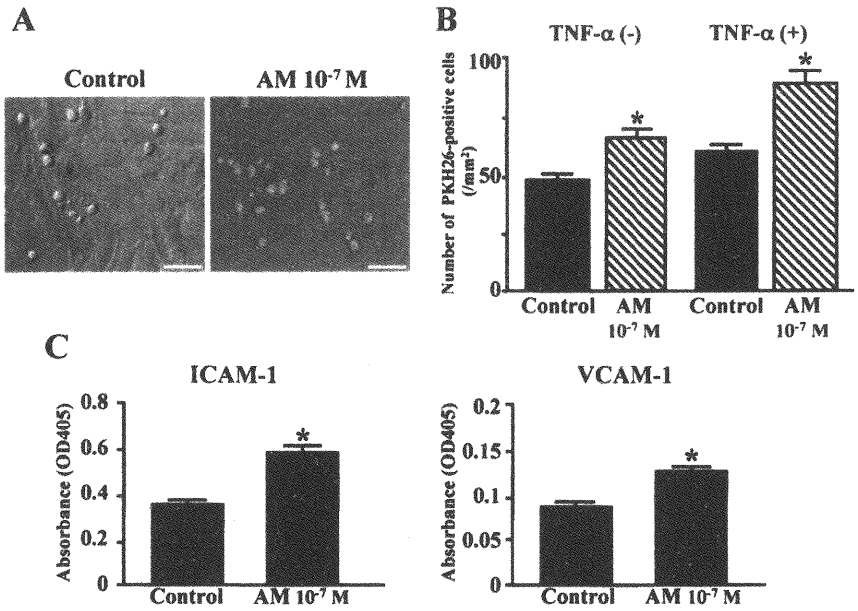

Figure 5. A and B, Adhesion assay. Representative photographs of red fluorescence–labeled MNC adhesion to HUVEC monolayer with and without AM (A). Quantitative analysis of MNC adhesion (B). Bars: 50 μm. C, Surface expression of ICAM-1 and VCAM-1 in HUVECs with or without AM. Data are mean±SEM. TNF indicates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

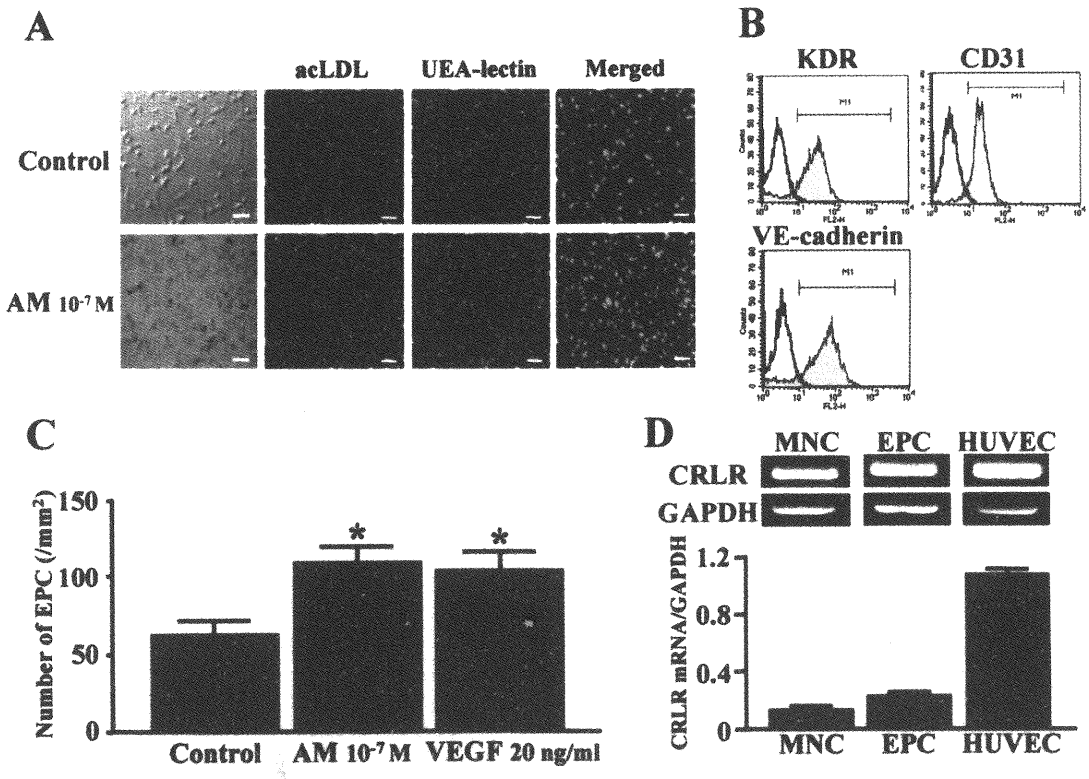

Figure 6. A through C, EPC culture assay. Cultured adherent cells took up DiI-acLDL (red) and FITC-labeled lectin (green) in same fields (A). Fluorescence-activated cell sorting analyses revealed that most adherent cells expressed KDR, VE cadherin, and CD31 (B). Culture of MNCs with AM significantly 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.

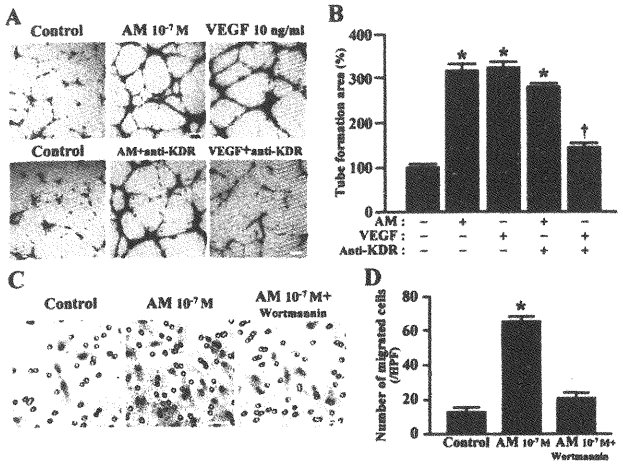

Figure 7. A and B, Matrigel assay. Representative photographs of tube formation (A). Quantitative analysis of tube formation area (B). Data are mean±SEM. *$P<0.01$ vs control; †$P<0.01$ vs VEGF. Bars: 20 μm. C and D, Migration assay. Representative photographs of migrated SMCs (C). Quantitative analyses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

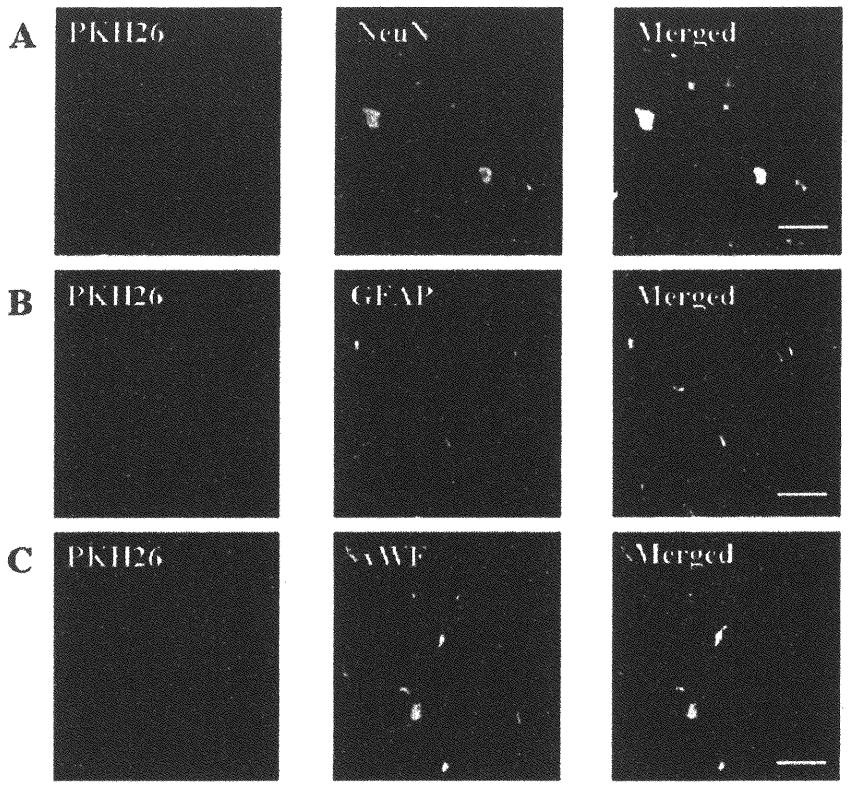

Figure 1. Engraftment and differentiation of transplanted MSCs. PKH26-labeled MSCs were frequently observed in ischemic penumbra. Some PKH26-positive MSCs (red) expressed neuronal marker (NeuN; green; A), astrocyte marker (GFAP; green; B), or endothelial cell marker (vWF; green; C). Bars=20 μm.

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.

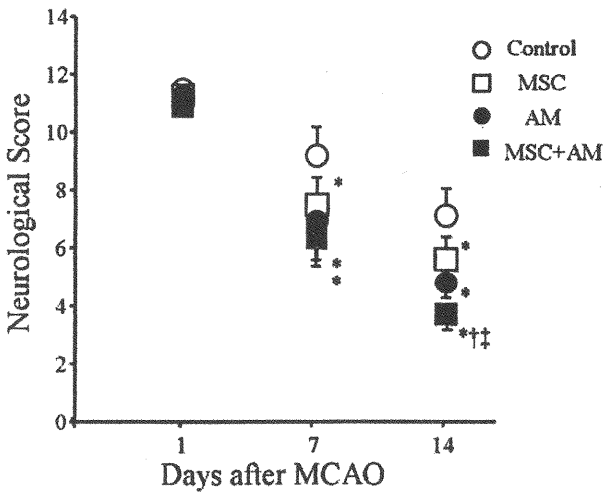

Figure 2. Neurological score on days 1, 7, and 14 in the control group, MSC group, AM group, and MSC+AM group. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs MSC group; ‡$P<0.05$ vs AM group.

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-

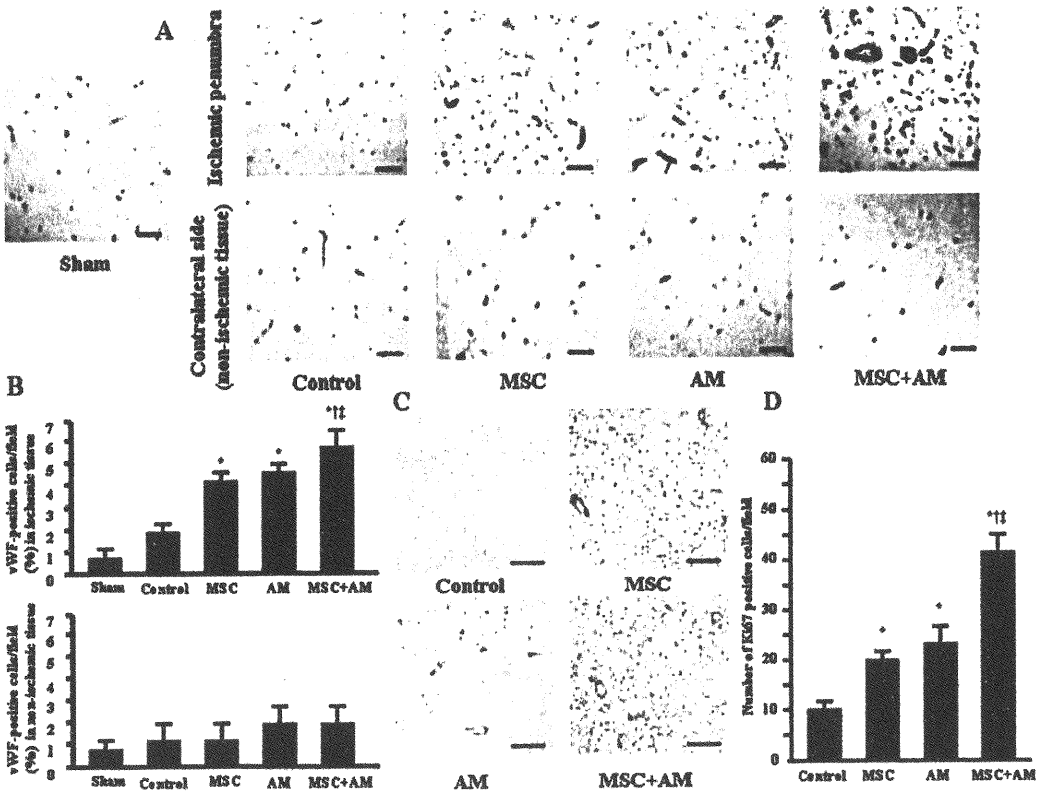

Figure 3. A, Representative photomicrographs of vWF staining in ischemic penumbra (top) and in contralateral nonischemic tissue (bottom). Bars=25 μm. B, Quantitative analysis of angiogenesis using the area of vWF staining in ischemic penumbra (top) and in nonischemic tissue (bottom). C, Representative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

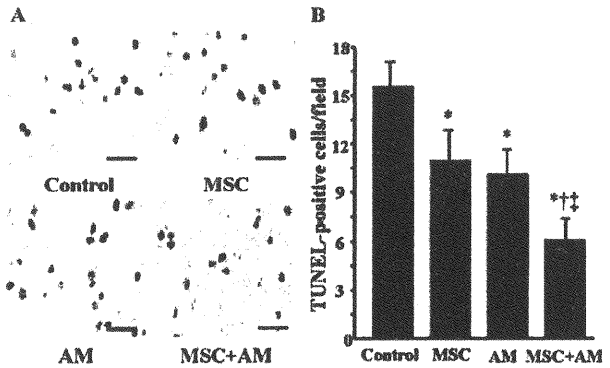

Figure 4. A, Representative photomicrographs of TUNEL staining in ischemic penumbra. The number of TUNEL-positive cells (DAB; brown) in the MSC+AM group was markedly lower than that in the other 3 groups. B, Quantitative analysis of the number of TUNEL-positive cells. Data are mean±SEM. *$P<0.05$ vs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

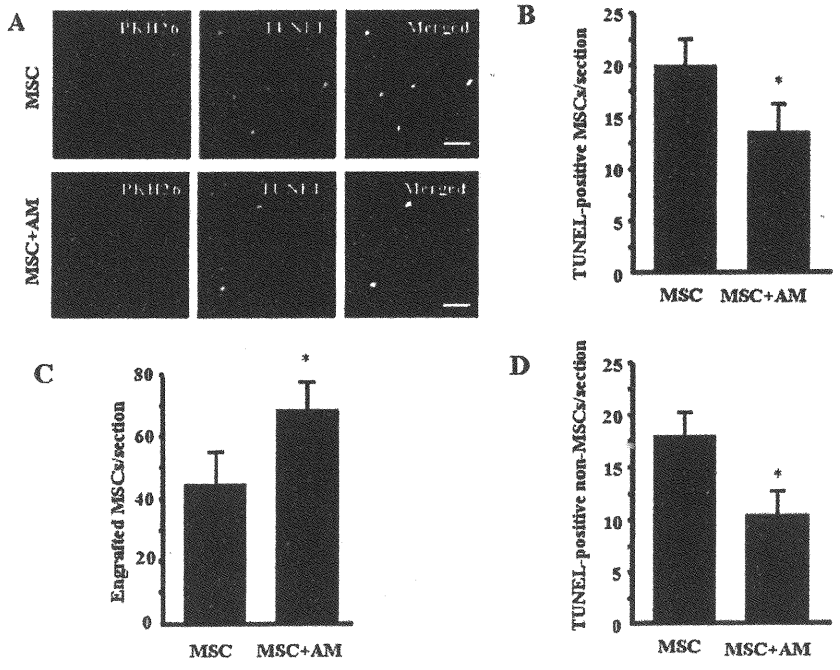

Figure 5. A, Representative photomicrographs of MSC apoptosis after transplantation. Transplanted MSCs were labeled with PKH26. TUNEL-positive cells (green) were frequently observed in ischemic penumbra. Infusion of AM decreased TUNEL-positive MSCs (double-positive cells, merged). B, Quantitative analysis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

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Adrenomedullin: angiogenesis and gene therapy

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Nagaya, Noritoshi, Hidezo Mori, Shinsuke Murakami, Kenji Kangawa, and Soichiro Kitamura. Adrenomedullin: angiogenesis and gene therapy. *Am J Physiol Regul Integr Comp Physiol* 288: R1432–R1437, 2005; doi:10.1152/ajpregu.00662.2004.—Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma. AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis, promotes angiogenesis, and affects vascular tone and permeability. The angiogenic effect of AM is mediated by activation of Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and focal adhesion kinase in endothelial cells. Both AM and its receptor, calcitonin receptor-like receptor, are upregulated through a hypoxia-inducible factor-1-dependent pathway under hypoxic conditions. Thus AM signaling plays an important role in the regulation of angiogenesis in hypoxic conditions. Recently, we have developed a nonviral vector, gelatin. Positively charged gelatin holds negatively charged plasmid DNA in its lattice structure. DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer. Administration of AM DNA-gelatin complexes induces potent angiogenic effects in a rabbit model of hindlimb ischemia. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of tissue ischemia. Endothelial progenitor cells (EPCs) play an important role in endothelial regeneration. Interestingly, EPCs phagocytose ionically linked DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs. AM gene transfer into EPCs inhibits cell apoptosis and induces proliferation and migration, suggesting that AM gene transfer strengthens the therapeutic potential of EPCs. Intravenous administration of AM gene-modified EPCs regenerate pulmonary endothelium, resulting in improvement of pulmonary hypertension. These results suggest that in vivo and in vitro transfer of AM gene using gelatin may be applicable for intractable cardiovascular disease.

regeneration; endothelium; ischemia; pulmonary hypertension

ADRENOMEDULLIN (AM) IS A POTENT, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma (36). The peptide consists of 52 amino acids with an intramolecular disulfide bond, sharing slight homology with calcitonin gene-related peptide and amylin. Immunoreactive AM is detected in plasma and a variety of tissues including, blood vessels, heart, and lungs (19). Particularly, AM shows a variety of effects on the vasculature that include vasodilatation (23), regulation of permeability (16), inhibition of endothelial apoptosis (31), and promotion of angiogenesis (1, 35, 60). In addition, AM has protective effects against vascular injury, including oxidative stress (33, 69, 84). It is becoming clear that either activation or disruption of AM signaling might contribute to many pathological conditions, including hypertension (22), congestive heart failure (55), pulmonary hypertension (29), neoplastic growth (39), and inflammatory disease (59). To date, the major biological acitivities of AM in vitro and in vivo are *1)* vasodilation, *2)* diuresis and natriuresis, *3)* positive inotropic effect, *4)* inhibition of endothelial cell apoptosis, *5)* induction of angiogenesis, *6)* inhibition of cardiomyocyte apoptosis, *7)* suppression of aldosterone production, *8)* anti-inflammatory activity, and *9)* antioxidant activity. We and others have demonstrated that intravenous administration of AM decreases systemic and pulmonary arterial pressure and induces diuresis and natriuresis (47, 52, 65), suggesting that AM is involved in the regulation of vascular tone and body fluid. Subsequent studies have demonstrated beneficial hemodynamic effects and direct cardioprotective effects of AM infusion in the treatment of congestive heart failure (57, 61–64).

Until recently, only vascular endothelial growth factor (VEGF) (80), fibroblast growth factor (68), platelet-derived growth factor (37), and angiopoietin (74) were known to have profound angiogenic effects. More recently, however, the angiogenic potential of AM has attracted investigators' attention (35, 41, 59, 81). A previous study has shown that vascular abnormalities are present in homozygous AM knockout mice (70), suggesting that AM is essential for vascular morphogenesis. AM activates the PI3K/Akt-dependent pathway in vascular endothelial cells (58), which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation (27). These findings raise the possibility that AM plays a role in modulating angiogenesis and neovascular-

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ization. This review focused on the angiogenic effects of AM and the therapeutic potential of AM gene transfer for the treatment of intractable cardiovascular disease.

ENDOGENOUS AM PRODUCTION IN ISCHEMIC CONDITIONS

Hypoxia (14, 53) and cytokine production (73) in ischemic heart disease or septic shock, as well as shear stress (7) in hypertension and heart failure induce AM secretion by vascular cells (Fig. 1). We have shown that plasma AM level is increased in patients with acute myocardial infarction (40, 49), peripheral arterial occlusive disease (75), and congestive heart failure (28, 55). Tissue levels of AM peptide and mRNA are also markedly increased in ischemic myocardium (18, 50) and failing heart (8, 56, 78, 82). These findings suggest that expression of AM is upregulated under tissue ischemia and inflammation, both of which are associated with neovascularization. An in vitro study has demonstrated that AM is upregulated through a hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions (14). Thus hypoxia/HIF-1 is one of the most potent regulators of AM production (Fig. 1). A recent study has demonstrated that heterozygous AM knockout mice [AM(+/−)] show significantly less blood flow recovery with less collateral capillary development than their wild-type mice (20). Administration of AM promotes blood flow recovery and capillary formation in AM(+/−) mice. These findings suggest that endogenous AM may play an important role in the regulation of angiogenesis under ischemic conditions. Considering the angiogenic potency of AM, increased endogenous AM represents a compensatory mechanism as an angiogenic factor promoting neovascularization under hypoxic conditions.

ANGIOGENIC EFFECTS OF AM AND ITS SIGNALING PATHWAY

AM signaling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis (31), promotes angiogenesis (35, 60), and affects vascular tone (23). Angiogenesis is a multistep process that involves migration and proliferation of endothelial cells, functional maturation of the newly assembled vessels, and remodeling of the extracellular matrix (26). Akt, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2), and focal adhesion kinase (p125FAK) play an important role in angiogenesis in endothelial cells. Kim et al. (35) demonstrated that AM activated Akt, MAPK/ERK1/2, and p125FAK in human umbilical vein endothelial cells (HUVECs), and produced increases in their DNA synthesis and migration. AM induced tube formation in HUVECs, and its effect was inhibited by pretreatment with a phosphatidylinositol 3′-kinase (PI3K) inhibitor or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)1/2 inhibitor. These findings suggest that AM exerts angiogenic activities through activation of Akt, MAPK, and p125FAK in endothelial cells (Fig. 1). In vivo, overexpression of AM augments collateral flow in ischemic tissues partly through activation of endothelial nitric oxide synthase (eNOS) (1). Earlier studies have shown that the vasodilatory effects of AM are mediated by cAMP/protein kinase in smooth muscle cells (SMCs) (23) and by the eNOS/NO pathway in endothelial cells (17). Thus AM-induced angiogenesis and vasodilation may synergistically improve blood perfusion in ischemic tissues.

Recently, a seven-transmembrane G-protein-coupled receptor, calcitonin receptor-like receptor (CRLR), and receptor activity modifying proteins (RAMPs) have been recognized as integral components of the AM signaling system (38, 43). CRLR has demonstrated the expression of the transcript predominantly in microvascular endothelial cells. This finding supports the view that CRLR is potentially a major mediator of the effects of AM on the vasculature. The effect of AM on CRLR is modified by RAMP2 and RAMP3. The angiogenic effect of AM is mediated by CRLR/RAMP2 and CRLR/RAMP3 receptors (Fig. 1). VEGF and AM act synergistically to induce angiogenic-related effects on endothelial cells in vitro (11). However, blocking antibodies to VEGF cannot significantly inhibit AM-induced capillary tube formation by

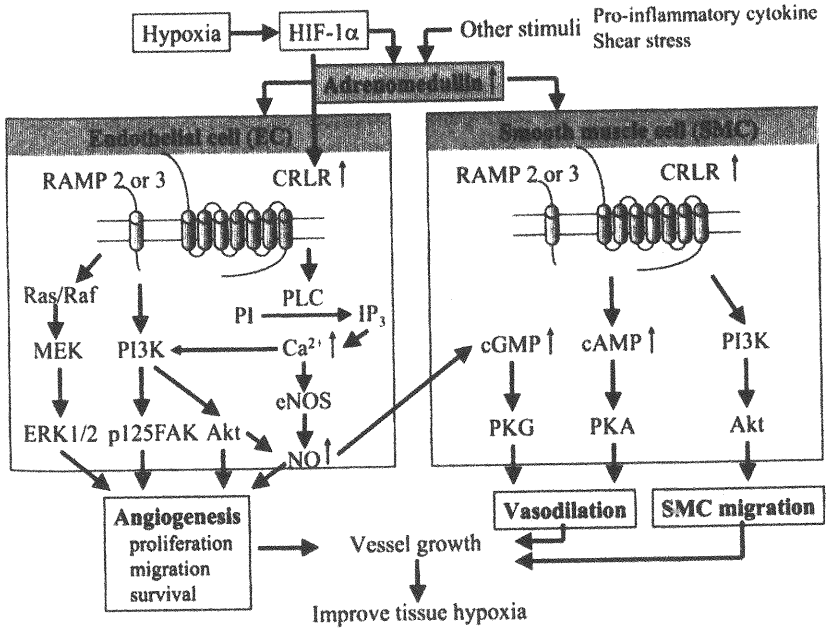

Fig. 1. Signaling pathway of adrenomedullin (AM) in vascular endothelial cells and smooth muscle cells. Both AM and calcitonin-receptor-like receptor (CRLR) are upregulated through a, hypoxia-inducible factor-1 (HIF-1)-dependent pathway under hypoxic conditions. AM binds to CRLR modified by receptor-activity-modifying protein 2 (RAMP2) and RAMP3. AM induces angiogenesis through activation of Akt, MAPK, and p125FAK in endothelial cells. AM also induces SMC migration and vasodilation. These activities synergistically improve tissue ischemia. MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; p125FAK, focal adhesion kinase; PLC, phospholipase C; PI, phosphatidylinositol; IP_3, inositol triphosphate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; cGMP, guanosine 3′,5′-cyclic monophosphate; PKG, protein kinase G; PKA, protein kinase A.