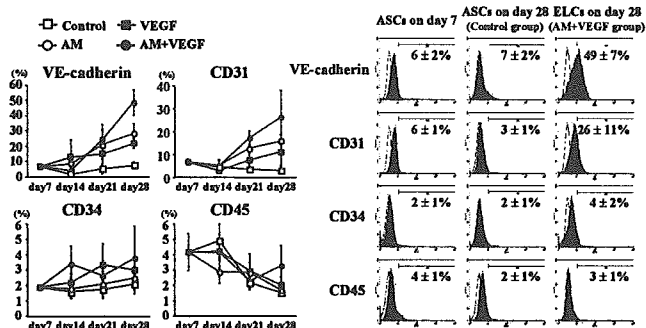


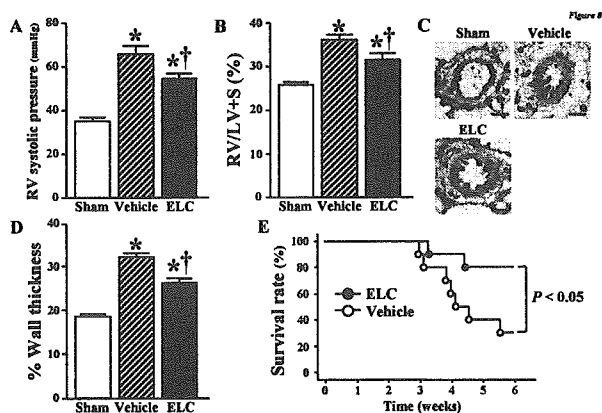
(図 1)



ラット肺高血圧モデルへの投与では MCT 投与の対照群に比して有意に血行動態の改善や右心重量比の減少、および毛細管密度の増加や肺動脈中膜厚の減少を認めた (図 2)。また、PKH 染色した移植細胞は 39%が肺組織中に取り込まれており、気管支壁 (60%)、肺動脈間質 (26%)、肺小動脈壁 (9%)、気管支動脈 (5%以下) に局在していた。

さらに 6 週間の生存率も有意に改善させた (図 2)。

(図 2)



#### D. 考察

今回の研究結果から、AM と VEGF を添加培養した脂肪由来前駆細胞は、①PI3K を介して血管内皮系へ一部分化すること、②血管再生能を有し、かつ脂肪になりにくい性質を有すること、③多くの血管新生因子を分泌すること、④経静脈的な移植で肺に取り込まれ血管新生を促すこと、⑤移植

治療で肺高血圧の病態と生存率を改善することが明らかとなった。

この細胞移植の効果は、移植細胞の分化能のみでなく、血管新生因子のパラクライン効果によるものと考えられた。細胞治療としてはこれまでに骨髄単核球、血管内皮前駆細胞、骨髄間葉系幹細胞が用いられてきたが、AM と VEGF により分化誘導した脂肪間質由来前駆細胞は、それらと同等以上の血管新生因子分泌能を持ち、再生医療の新たな細胞源として有用である可能性が示された。

#### E. 結論

AM と VEGF を添加培養した脂肪由来前駆細胞は、PI3K を介して血管内皮系へ分化し、高い血管再生能を有し、かつ脂肪になりにくい性質を持つ。また、経静脈的移植でラット肺高血圧モデルの病態と生存率を改善することから、新たな肺高血圧治療法として有用である可能性が示唆された。

#### F. 健康危険情報

なし。

#### G. 研究発表

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## H. 知的財産権の出願・登録状況（予定を含む）

### 1. 特許取得

特願 2005-117588

### 2. 実用新案登録

なし。

### 3. その他

研究協力者

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## 別紙 4

## 研究成果の刊行に関する一覧表レイアウト

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工業所有権の種類: 特許

番号: 出願番号 特願2005-111889 (出願日: 平成17年4月8日)

2. PAMP: 発明の名称 「心筋障害の予防又は治療剤」

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出願人: 国立大学法人宮崎大学

工業所有権の種類: 特許

番号: 出願番号 特願2005-114786 (出願日: 平成17年4月12日)

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

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

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

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

**Key Words:** peptides ■ angiogenesis ■ peripheral vascular disease

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

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.<sup>5</sup> Previous studies have reported that abnormalities of vascular structure are present in homozygous AM knockout mice.<sup>6,7</sup> A recent study has demonstrated that blood

flow recovery in ischemic limb and tumor angiogenesis are substantially impaired in heterozygous AM knockout mice.<sup>8</sup> Furthermore, AM has been shown to inhibit vascular endothelial cell apoptosis and induce angiogenesis through the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.<sup>9,10</sup> These results suggest that AM is indispensable for modulating angiogenesis and vasculogenesis. When these findings are taken together, combination therapy with MNC transplantation and AM infusion may have additional or synergetic effects on therapeutic angiogenesis for the treatment of severe peripheral vascular disease. Thus, the purposes of the present study were (1) to investigate whether local infusion of AM enhances the angiogenic potency of MNC transplantation in a rat model of hindlimb ischemia and (2) to investigate the effects of AM on the survival, adhesion, and differentiation of transplanted MNCs.

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## Methods

### Animal Model of Hindlimb Ischemia

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

### MNC Transplantation and AM Infusion

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

### Assessment of Blood Perfusion

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

### Histological Assessment

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

### Monitoring of Transplanted MNCs in Ischemic Hindlimb Muscle

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

### In Situ Detection of MNC Apoptosis

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

### In Vitro Apoptosis Assay

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

### Adhesion Assay

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

### Cell ELISA

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

### EPC Culture Assay

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

### Fluorescence-Activated Cell Sorting Analysis

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

### Real-Time Polymerase Chain Reaction

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

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

### In Vitro Matrigel Assay

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

### Measurements of Cytokines

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

### Migration Assay

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

### Statistical Analysis

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

## Results

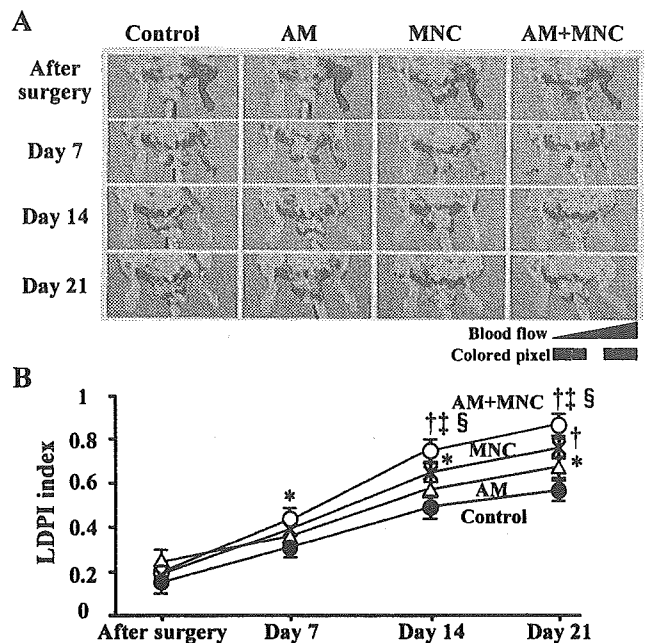
### Blood Perfusion and Capillary Density

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

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

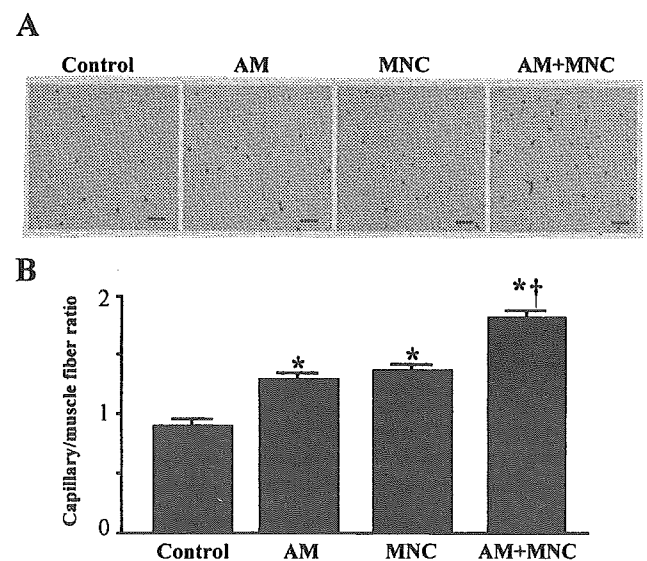
### Differentiation of Transplanted MNCs

Three weeks after MNC transplantation, PKH26-labeled MNCs were frequently observed in the AM+MNC group, and these transplanted cells were positive for vWF (Figure 3A). Most of these cells were also stained by CD31 (data not shown). The number of PKH26/vWF double-positive cells was significantly higher in the AM+MNC group than in the

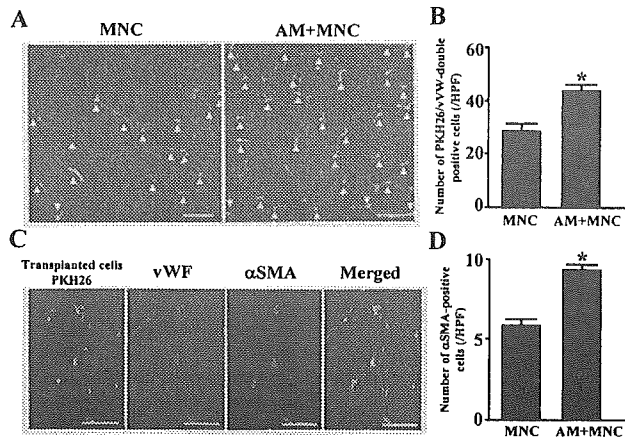


**Figure 1.** A, Representative examples of serial laser Doppler perfusion images. Blood perfusion of ischemic hindlimb increased notably in AM+MNC group (red to yellow). B, Quantitative analysis of hindlimb blood perfusion with LDPI index, ratio of ischemic to nonischemic hindlimb blood perfusion. Data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

MNC group (Figure 3B). Although PKH26/ $\alpha$ -SMA double-positive cells were not detected in ischemic muscle of each group, newly formed vascular structures in the AM+MNC group included  $\alpha$ -SMA-positive cells (Figure 3C). The number of  $\alpha$ -SMA-positive cells in the MNC-derived vascular structures was significantly higher in the AM+MNC group than in the MNC group (Figure 3D).



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



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

### Antiapoptotic Effect of AM on MNCs

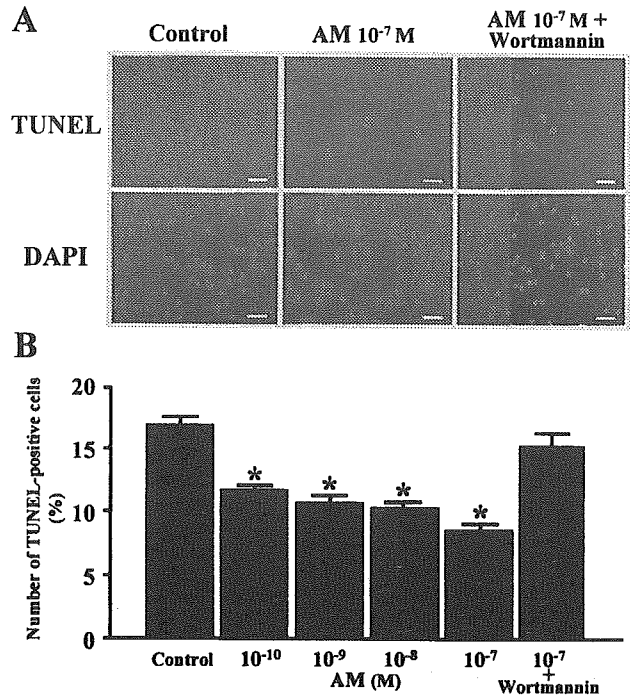
In vitro, serum starvation induced MNC apoptosis, as indicated by detection of TUNEL-positive cells (Figure 4A). When incubated in the presence of AM, the percentage of TUNEL-positive cells markedly decreased in a dose-dependent manner (Figure 4B). However, pretreatment with wortmannin, a PI3K inhibitor,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

### Effect of AM on MNC Adhesiveness

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

### Effect of AM on EPC Expansion

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



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

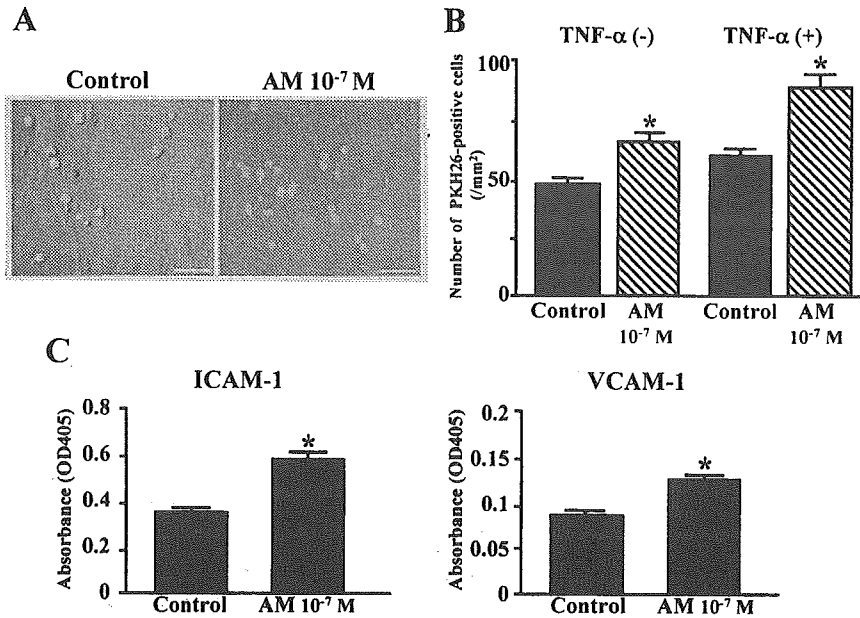
CRLR mRNA was highest in HUVECs, followed by EPCs and MNCs.

### Effects of AM on Tube Formation and SMC Migration

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

### Discussion

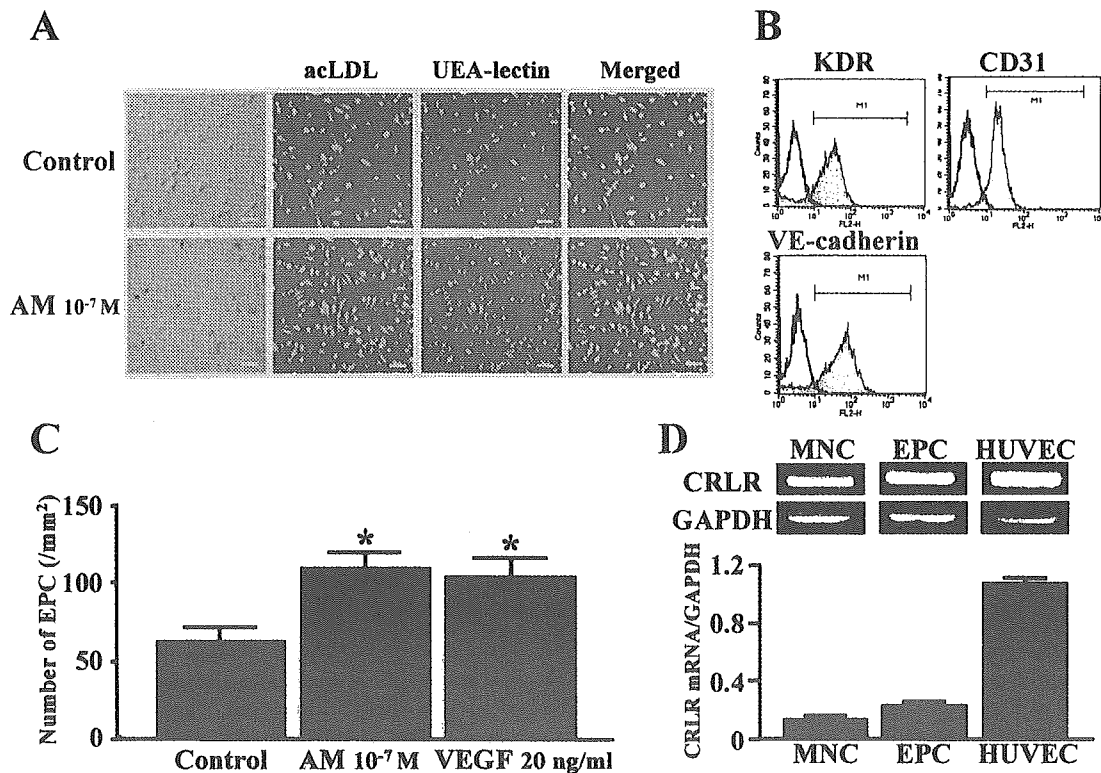
In the present study, we demonstrated in vivo that AM infusion or MNC transplantation alone induced angiogenesis in a rat model of hindlimb ischemia, the combination of AM infusion and MNC transplantation enhanced MNC-induced angiogenesis, and AM increased the number of MNC-derived vWF-positive cells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



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

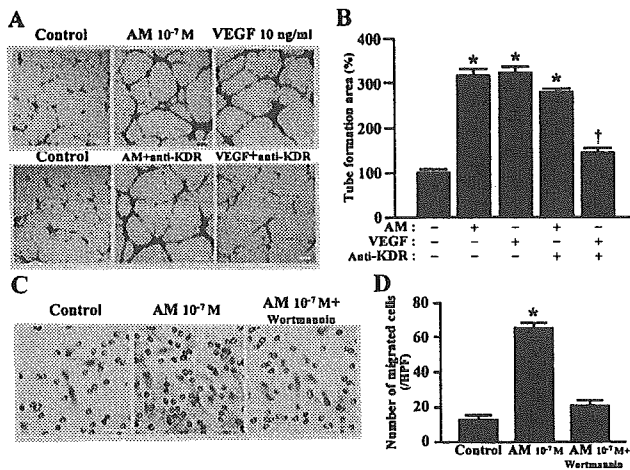
MNC transplantation causes therapeutic angiogenesis by supplying EPCs and multiple angiogenic cytokines such as VEGF.<sup>3,4</sup> The present study showed that local infusion of AM significantly increased blood perfusion and capillary density in ischemic hindlimb muscle. Furthermore, a combination of AM infusion and MNC transplantation significantly increased blood perfusion and capillary den-

sity 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.<sup>10,18</sup> 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 Dil-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  $\pm$  SEM. \* $P < 0.01$  vs control. Bars: 50  $\mu$ m. 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 $\pm$ SEM. \* $P$ <0.01 vs control; † $P$ <0.01 vs VEGF. Bars: 20  $\mu$ m. C and D, Migration assay. Representative photographs of migrated SMCs (C). Quantitative analyses of SMC migration (D). Data are mean $\pm$ SEM. \* $P$ <0.01 vs control. Bars: 50  $\mu$ m.

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.<sup>19</sup> 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.<sup>20</sup> 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.<sup>21</sup> 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.<sup>13,22</sup> 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.<sup>23</sup> We demonstrated in vivo that local infusion of AM increased the number of  $\alpha$ -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.<sup>6,7</sup> 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.<sup>24</sup> Thus, combination therapy with AM infusion and MNC transplantation may be a novel and promising therapeutic strategy for the treatment of severe peripheral vascular disease.

## Conclusions

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

## Acknowledgments

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

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

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

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

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

**Key Words:** angiogenesis ■ apoptosis ■ stroke

Despite the advances in medical and surgical treatment, stroke is still a major cause of morbidity and mortality. Mesenchymal stem cells (MSCs) are multipotent, and some transplanted MSCs can differentiate into neuronal cells and endothelial cells in the recipient brain.<sup>1</sup> A recent study has shown that MSCs have ability to pass blood-brain barrier, particularly in injury sites.<sup>1-3</sup> In addition, transplantation of MSCs into the brain of experimental stroke animals has been shown to improve neurological functional recovery.<sup>1,3</sup> The effect of MSC transplantation is dependent on the number of transplanted MSCs.<sup>1</sup> However, the viability of MSCs after transplantation is relatively poor.<sup>4</sup> 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.<sup>5</sup>

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

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## Materials and Methods

### Stroke Model

Male Lewis rats (Japan SLC, Hamamatsu, Japan) weighing 230 to 260 g were used in all experiments. Middle cerebral artery occlusion (MCAO) was performed by an intraluminal thread as described previously.<sup>2</sup> 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.<sup>9</sup> In brief, we euthanized male Lewis rats and harvested bone marrow. Bone marrow cells were introduced into 100-mm dishes and cultured in  $\alpha$ -minimum essential medium (MEM) 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,  $\approx$ 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.<sup>10</sup> Most of cultured adherent cells were positive for CD29 (98 $\pm$ 1%) and CD90 (99 $\pm$ 1%) and negative for CD34 (2 $\pm$ 1%) and CD45 (1 $\pm$ 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 $\pm$ 14 pg/10<sup>6</sup> 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 $\times$ 10<sup>6</sup> cells) suspended in PBS were injected via a tail vein. Four rats underwent a sham operation without an intraluminal thread. AM (0.05  $\mu$ g/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.<sup>8</sup>

### Detection of MSC Differentiation in Ischemic Hemisphere

Red fluorescent-labeled MSCs were transplanted to examine MSC differentiation as described previously.<sup>11</sup> 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  $\approx$ 100%, and transplanted cells are detectable at least up to 4 months after transplantation in the host brain.<sup>11</sup> 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- $\mu$ m-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 isothiocyanate (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.<sup>1</sup> 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,<sup>1</sup> 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 streptavidin-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,<sup>2</sup> and images ( $\times$ 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.<sup>12</sup> The values corresponding to total brown areas were averaged and expressed as the mean percentage of stained vessel area per 100  $\mu$ m<sup>2</sup>. 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.

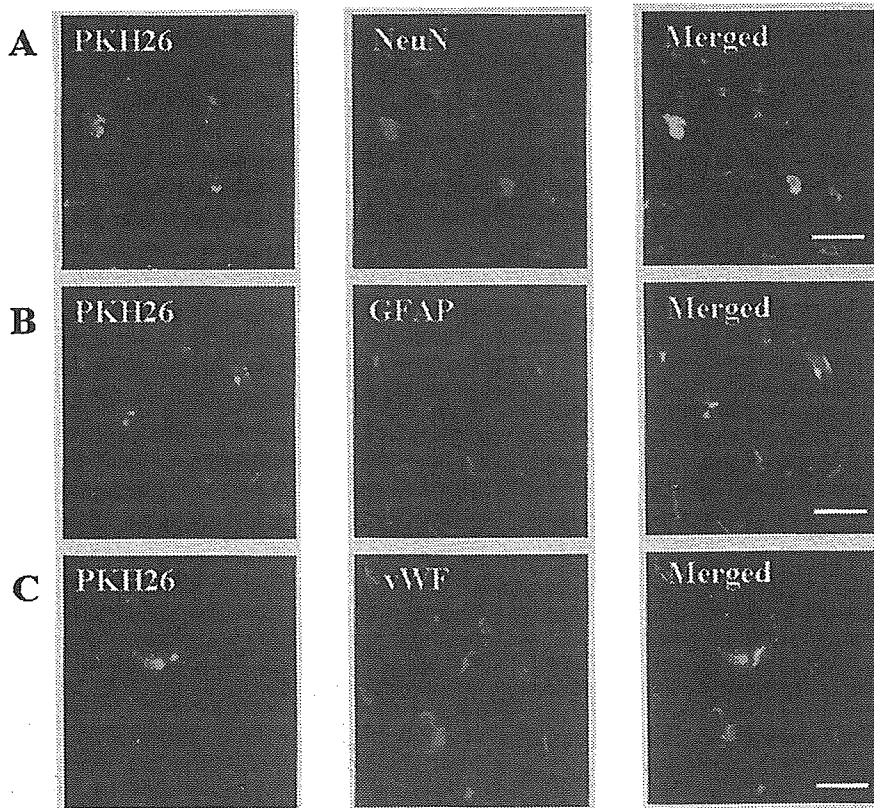
### Statistical Analysis

All data were expressed as mean $\pm$ 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

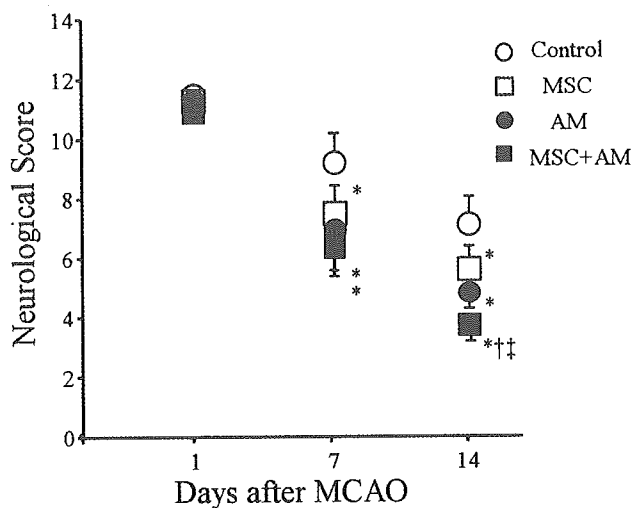


**Figure 1.** Engraftment and differentiation of transplanted MSCs. PKH26-labeled MSCs were frequently observed in ischemic penumbra. Some PKH26-positive MSCs (red) expressed neuronal marker (NeuN; green; A), astrocyte marker (GFAP; green; B), or endothelial cell marker (vWF; green; C). Bars=20  $\mu$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

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



**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  $\pm$  SEM. \* $P$ <0.05 vs control group; † $P$ <0.05 vs MSC group; ‡ $P$ <0.05 vs AM group.

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.

### 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

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

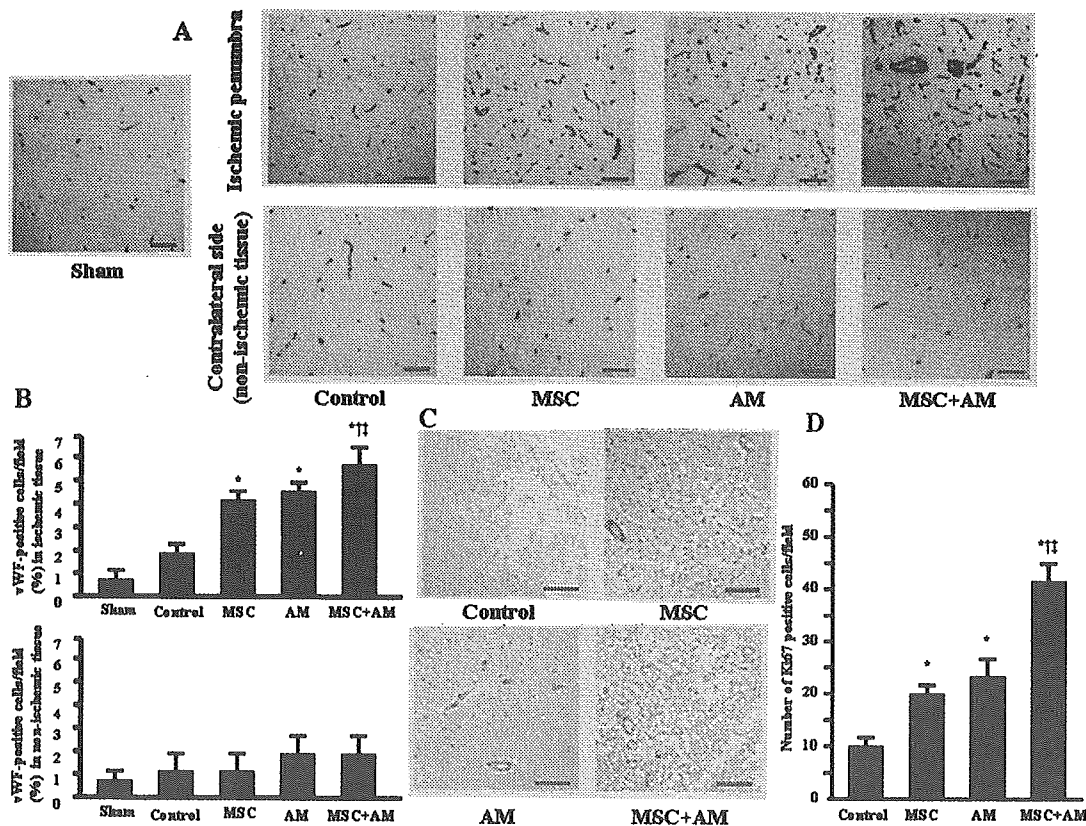
Angiogenesis in the ischemic penumbra was observed after MCAO compared with sham operation (Figure 3A). Furthermore, MSC transplantation or AM infusion induced angiogenesis in the ischemic penumbra, and particularly, the angiogenic effect was marked after combined therapy of MSCs and AM. Quantitative 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.