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 ofangiogenesis. (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. A recent study has shown that MSCs have ability to pass blood–brain barrier, particularly in injury sites. In addition, transplantation of MSCs into the brain of experimental stroke animals has been shown to improve neurological functional recovery. The effect of MSC transplantation is dependent on the number of transplanted MSCs. However, the viability of MSCs after transplantation is relatively poor. 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 vasodialatory peptide that was originally isolated from human pheochromocytoma.

Recent study has shown that intramuscular administration of AM DNA induces therapeutic angiogenesis in a hindlimb ischemic model via activation of Akt. 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.
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. 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. In brief, we euthanized male Lewis rats and harvested bone marrow. Bone marrow cells were introduced into 100-mm dishes and cultured in a 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, ~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. 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.

Detection of MSC Differentiation in Ischemic Hemisphere
Red fluorescent-labeled MSCs were transplanted to examine MSC differentiation as described previously. In brief, suspended MSCs were labeled with fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit; Sigma). Three minutes after labeling, FBS was added for 3 minutes. These cells were analyzed by fluorescence-activated cell sorting as described previously. 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.

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 2-mm coronal blocks, and each section was stained with hematoxylin and eosin. Infarct size was determined by the “indirect method,” as described previously, 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, 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. The values corresponding to total brown areas were averaged and expressed as the mean percentage of stained vessel area per 100 μm². 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...
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.

**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

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Day 1</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>31±1</td>
<td>31±2</td>
</tr>
<tr>
<td>MSC</td>
<td>8</td>
<td>27±1*</td>
<td>29±2</td>
</tr>
<tr>
<td>AM</td>
<td>8</td>
<td>28±1*</td>
<td>29±1</td>
</tr>
<tr>
<td>MSC+AM</td>
<td>8</td>
<td>25±1†‡</td>
<td>28±2</td>
</tr>
</tbody>
</table>

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.
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.](http://stroke.ahajournals.org/)
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 anti-apoptotic 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. A previous report has demonstrated that pretreatment with AM reduces brain injury and improves neurological deficits in a rat stroke model. 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. 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 anti-apoptotic effects on various cells through the PI3K/Akt pathway. 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. The beneficial effects are considered to be mediated by increases in endogenous angiogenic and anti-apoptotic factors including VEGF, a potent neuroprotective factor, and by differentiation of MSCs themselves into neuronal cells. 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 anti-apoptotic factors. Nevertheless, the majority of transplanted MSCs were positive for TUNEL staining on day 3. Interestingly, infusion of AM significantly decreased the number of TUNEL-positive cells in the ischemic penumbra.
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 synergistic effects on MSC transplantation, leading to further improvement in neurological functions after stroke. Interestingly, a significant increase in body weight was observed in rats with low neurological score after treatment. A previous report has shown that body weight after stroke was higher in bFGF-treated rats than in vehicle-treated rats.15 These results suggest that earlier recovery of neurological deficits might have restored impaired food intake after stroke.

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

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

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References
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